Institute of Biotechnology

and

Division of Pharmacology and Pharmacotherapy
Faculty of Pharmacy

and

Doctoral School in Health Sciences
Doctoral Programme in Drug Research
University of Helsinki

GDNF and Neurturin isoforms in an experimental model of Parkinson's disease

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Pharmacy, University of Helsinki in Auditorium 2, Infocenter Korona, Viikki, on September 23rd 2017, at 10 am

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ISBN 978-951-51-3633-6 (paperback)
ISBN 978-951-51-3634-3 (PDF, https://ethesis.helsinki.fi)
ISSN 2342-3161 (Print)
ISSN 2342-317X (Online)
Unigrafia
Helsinki 2017

Du stannar på en borderline
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Håkan Hellström

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease characterized by intracellular proteinaceous inclusions called Lewy bodies and progressive loss of dopaminergic neurons in the substantia nigra (SN). The first symptoms of PD are non-motor, such as hyposmia and gastrointestinal disturbances, followed by motor symptoms, such as tremor and rigidity. Currently available therapies, medication, surgical procedures and supportive therapies, are symptomatic and do not affect the underlying cause of the disease — the neuronal degeneration. Thus, a new therapy which would restore the dopaminergic phenotype of dying neurons and thus slow down or even halt the progress of the disease is needed.

Neurotrophic factors are secretory proteins regulating survival and functioning of the neurons as well as the formation of new neuronal contacts. Neurotrophic factors have shown great potential in animal models of PD, but in clinical trials, the results have been contradictory. One possible explanation for this is poor diffusion and bioavailability of the therapeutic proteins in the target tissue. The aim of this study was to explore the neuroprotective effects of the isoforms of two of the most potent dopamine neurotrophic factors, GDNF (glial cell line-derived neurotrophic factor) and its homolog neurturin (NRTN) in an experimental model of PD, and to characterize a new stable low-dose 6-hydroxydopamine (6-OHDA) rat PD model.

In the PD model the degeneration of the nigrostriatal pathway was induced by administrating toxic dopamine analog 6-OHDA into the striatum, where the nerve terminals of the dopaminergic neurons are located. We compared several different administration paradigms to find the optimal parameters to induce a stable lesion model with high success rate. The cell loss induced with low doses (6-9 µg) of 6-OHDA was at similar level as the cell loss induced with higher (20 µg) doses of 6-OHDA. The advantage of using low 6-OHDA doses is the avoidance of non-specific damage, which occurs with higher 6-OHDA doses. Moreover, the low-dose induced lesions have high success rate, reducing the number of animals needed in the experiments and increasing the reliability of the obtained results.

The spreading of NRTN in the brain tissue was improved by modifying the extracellular matrix binding sequence of the protein. New NRTN variants were biologically active and were able to initiate signaling via tyrosine kinase Ret (rearranged during transfection). In the neuroprotection assay in rat 6-OHDA model of PD NRTN variant N4 protected the dopaminergic neurons in the SN and fibers in the striatum as well as improved the motor behavior of the animals. In neurorestoration assay, N4 showed a trend in improving the behavioral deficits of the animals. GDNF, on the other hand, was administered to the brain with viral vectors, enabling long-term protein expression in the target tissue. GDNF has been widely studied, but the research has focused on the full-length constitutively secreted α-isoform, whereas the biology of the shorter and activity-dependently secreted β-GDNF has not been studied in vivo before. In the non-lesioned striatum, both isoforms increased striatal dopamine transporter-immunoreactivity. Both isoforms also protected the dopaminergic neurons in SN from 6-OHDA-induced degeneration.

The results show that these new and less studied neurotrophic factor isoforms are able to slow down the degeneration of the midbrain dopaminergic neurons. In other words, both NRTN variant N4 and β-GDNF are potential disease-modifying factors for PD.

TIIVISTELMÄ

Parkinsonin tauti on etenevä hermorappeumasairaus, jossa substantia nigran dopamiinia tuottavat hermosolut tuhoutuvat. Parkinsonin taudin ensimmäiset oireet ovat nonmotorisia, kuten hajuaistin tai suoliston toimintahäiriöitä, joita seuraavat motoriset oireet, kuten lepovapina ja raajojen jäykkyys. Tällä hetkellä tarjolla olevat hoitomuodot, lääkehoito, kirurginen toimenpide ja fysioterapia, ainoastaan lievittävät Parkinsonin taudin oireita, mutta ne eivät pysäytä tai hidasta oireiden syynä olevaa hermosolujen tuhoutumista. Uusi terapia, joka palauttaisi rappeutuvien hermosolujen dopaminergisen fenotyypin ja siten hidastaisi tai jopa pysäyttäisi taudin etenemisen, mullistaisi Parkinsonin taudin hoidon.

Hermokasvutekijät ovat elimistön omia proteiineja, jotka säätelevät hermosolujen eloonjäämistä, uusien kontaktien muodostamista ja hermosolujen toimintaa. Hermokasvutekijät ovat osoittautuneet potentiaalisiksi uusiksi lääkemolekyyleiksi Parkinsonin taudin eläinmalleissa, mutta positiiviset tulokset eivät ole toistuneet kliinisissä kokeissa. Yhtenä syynä tähän on pidetty proteiinien heikkoa leviämistä kohdekudoksessa. Tämän tutkimuksen tarkoituksena oli tutkia kahden hermokasvutekijän, gliasolulinjaperäisen hermokasvutekijän GDNF:n (engl. glial cell line-derived neurotrophic factor) ja sen sukulaisproteiinin neurturiinin (NRTN) eri muotojen kykyä suojata keskiaivojen dopamiinia tuottavia hermosoluja Parkinsonin taudin kokeellisessa eläinmallissa sekä karakterisoida matalalla 6-hydroksidopamiini(6-OHDA)annoksella indusoitu Parkinsonin taudin eläinmalli.

Eläinmallissa nigrostriataalisen hermoradan osittainen tuhoutuminen aiheutettiin annostelemalla hermovälittäjäaine dopamiinin myrkyllistä analogia, 6-OHDA:a, striatumiin, jossa dopamiinia tuottavien hermosolujen hermopäätteet sijaitsevat. Vertailemalla erilaisia injektioasetuksia löysimme tavan, jolla annosteltuna hermosolujen tuho oli pysyvä ja toistettava. Matalilla 6-OHDA annoksilla (6-9 µg) aiheutettu solutuho osoittautui yhtä suureksi kuin suurilla annoksilla (20 µg) aiheutettu solutuho. Pienten 6-OHDA-annosten aiheuttaman solutuhon on kuitenkin osoitettu olevan spesifisempi kuin suurten 6-OHDA-annosten aiheuttama solutuho. Toistettava eläinmalli vähentää kokeissa käytettävien eläinten lukumäärää ja lisää tulosten luotettavuutta.

NRTN:n leviämistä kudoksessa parannettiin muokkaamalla jaksoa, jolla neurturiini sitoutuu soluväliaineeseen. Uudet neurturiinivariantit ovat biologisesti aktiivisia ja voivat aloittaa signaalivälityksen. 6-OHDA-eläinmallissa aivoihin annosteltu NRTN-variantti N4 suojasi substantia nigran dopamiinia tuottavia hermosoluja ja niiden hermopäätteitä striatumissa sekä kohensi eläinten motorista toimintakykyä. Myös myöhemmin annosteltuna N4 osoitti potentiaalia palauttaa eläinten motorinen toimintakyky. GDNF sen sijaan annosteltiin aivoihin virusvektorin avulla, mahdollistaen terapeuttisen proteiinin pitkäkestoisen tuotannon kohdekudoksessa. GDNF on laajalti tutkittu proteiini, mutta tutkimus on keskittynyt vain täyspitkään ja jatkuvasti soluista erittyvään α-muotoon ja lyhyempi, aktiivisuusriippuvaisesti soluista erittyvä β-muoto on vaikutuksiltaan tuntemattomampi. Ehjään striatumiin annosteltuna kumpikin muoto lisäsi dopamiinikuljettajaimmunoreaktiivisuutta striatumissa. Lisäksi kumpikin muoto suojasi keskiaivojen dopamiinia tuottavia hermosoluja 6-OHDA:n aiheuttamalta tuhoutumiselta.

Tulokset osoittavat uusien ja vähemmän tutkittujen hermokasvutekijämuotojen hidastavan keskiaivojen dopamiinia tuottavien hermosolujen tuhoutumista. Toisin sanoen, sekä neurturiinivarianti N4 että β-GDNF ovat potentiaalisia terapeuttisia proteiineja Parkinsonin taudin hoitoon.

ABBREVIATIONS

α-GDNF	pre-α-pro-GDNF
β-GDNF	pre-β-pro-GDNF
6-OHDA	6-hydroxydopamine
AAV	Adeno-associated virus
ANOVA	Analysis of variance
ATF6	Activating transcription factor 6
DAT	Dopamine transporter
DBS	Deep brain stimulation
ER	Endoplasmic reticulum
GABA	γ-aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFP	Green fluorescent protein
GFRα	GDNF receptor α
GP	Globus pallidus
GPe	Globus pallidus externa
GPi	Globus pallidus interna
GRP78	Glucose regulated protein 78
IRE1α	Inositol-requiring enzyme 1α
L-DOPA	L-dihydroxyphenylalanine, levodopa
LPS	Lipopolysaccharide
MFB	Medial forebrain bundle
MPP^+	1-methyl-4-phenyl-2,3-tetrahydropyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	mammalian target of rapamycin
Nrf2	Nuclear factor erythroid-2-related factor 2
NRTN	Neurturin
PD	Parkinson's disease
PERK	Double stranded RNA-activated protein kinase-like ER kinase
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1-α
PINK1	PTEN-induced kinase 1
Ret	Rearranged during transfection
ROS	Reactive oxygen species
scAAV1	Self-complementary adeno-associated virus, serotype 1
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TFEB	Transcription factor EB
TH	Tyrosine hydroxylase
UPR	Unfolded protein response
UPS	Ubiquitin-proasome system
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
WT	Wildtype

ORIGINAL PUBLICATIONS

The thesis is based on the following original publications:

I Runeberg-Roos P, Piccinini E*, Penttinen A-M*, Mätlik K, Heikkinen H, Kuure S, Bespalov MM, Peränen J, Garea-Rodríguez E, Fuchs E, Airavaara M, Kalkkinen N, Penn R, Saarma M. (2016) Developing therapeutically more efficient Neurturin variants for treatment of Parkinson's disease. Neurobiol Dis 96: 335-345

II Penttinen A-M, Suleymanova I*, Albert K*, Anttila J, Voutilainen MH, Airavaara M. (2016) Characterization of new low-dose 6-hydroxydopamine model of Parkinson's disease in rat. J Neurosci Res 94: 318-328

III Penttinen A-M, Koskela M, Voutilainen MH, Bäck S, Richie CT, Harvey BK, Tuominen RK, Nevalaita L, Saarma M, Airavaara M. Neuroprotective effects of pre-α-pro-GDNF and pre-β-pro-GDNF isoforms in rat 6-OHDA model of Parkinson's disease. Manuscript.

*equal contribution

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1 INTRODUCTION

200 years ago, in his essay “An essay on the shaking palsy”, James Parkinson described patients with a progressive disease manifested in various motor impairments, such as tremulous motion and bowed posture, and non-motor indications, such as sleep and gastrointestinal disturbances. The malady, which rarely seemed to affect individuals under fifty years old, had a long duration and therefore the different symptoms in each stage of the disease were difficult to connect. Thus, continued observation of the patient was needed to confirm the diagnosis. The origin of the disease was a puzzle, and so was the possible treatment. Nevertheless, Parkinson was optimistic about finding a remedy for the disease, at least to stop the progress of it, if not fully reversing it, and warned about considering shaking palsy as an incurable disease (Parkinson, 2002).

A long time has passed since James Parkinson’s suggestions of using mercury or diminishing inordinate actions of vessels in the diseased tissues (Parkinson, 2002), but there still is not a cure for shaking palsy, or Parkinson’s disease (PD) as it is currently known. All the available therapies at the moment are symptomatic, relieving the symptoms of the patients, but without affecting the progress of the disease (Oertel and Schulz, 2016). The main reason for the lack of effective therapy is the lack of understanding of the molecular mechanisms of PD. Most PD cases are idiopathic, age being the biggest risk factor. Also, genes, environmental factors, and lifestyle have been associated with PD. How these factors induce pathogenic cellular events, such as accumulation of fibrillar α-synuclein and the formation of Lewy bodies or mitochondrial dysfunction disturbing the cellular homeostasis leading to degeneration of midbrain dopaminergic neurons, is not fully understood (de Lau and Breteler, 2006; Lill, 2016; Przedborski, 2017). Hence, more knowledge of these mechanisms is needed to develop a disease-modifying therapy, which would slow down the progress of the disease.

In the 1950s, soluble tumor agent was observed to enhance the growth of the developing nerve cells and in fact, to be essential for the survival and development of the neurons (Levi-Montalcini, 1952). Survival-promoting effects of neurotrophic factors make them attractive candidates for the therapy of neurodegenerative diseases (Lin et al., 1993). Several neurotrophic factor families have been established, glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) consisting of GDNF, neurturin (NRTN), artemin, and persephin, being one of them. GFLs have been extensively studied and they have shown great potential in preclinical models of PD. However, translating the positive results from animal models to human patients has proven to be difficult (Bartus and Johnson, 2017a; Bartus and Johnson, 2017b; Kirik et al., 2017).

2 REVIEW OF THE LITERATURE

2.1 Parkinson's disease and degeneration of dopaminergic neurons

Dopamine was suggested to be an independent neurotransmitter in the brain in the late 1950s by Arvid Carlsson (Carlsson et al., 1957; Carlsson et al., 1958; Carlsson, 1959). Since Carlsson's observations, dopamine has been linked to reward and motivation, motor control, and regulation of endocrine functions (Björklund and Dunnett, 2007; Wise, 2008; Tekin et al., 2014). Hence, dysregulation of dopamine is associated with several neurological and psychiatric disorders, e.g. PD, schizophrenia, depression, and addiction (Björklund and Dunnett, 2007; Wise, 2008). PD is diagnosed based on the motor symptoms which appear after the loss of midbrain dopaminergic neurons exceeds a certain threshold. However, when, where, or why exactly this degenerative process starts, is not known (Braak et al., 2006; Engelender and Isacson, 2017).

2.1.1 Neurobiology of dopamine and its pathways

Dopamine is synthesized from amino acid tyrosine by conversion to L-3,4-dihydroxyphenylalanine (L-DOPA) (Nagatsu et al., 1964) and decarboxylated further to dopamine by L-amino acid decarboxylase. Dopamine synthesis is regulated by tyrosine hydroxylase (TH), which catalyzes the rate-limiting step of the dopamine synthesis, conversion of tyrosine to L-DOPA (Levitt et al., 1965). In the cell body TH is diffusely distributed in the cytoplasm with some association with endoplasmic reticulum (ER) and Golgi complex (Pickel et al., 1976). In contrast, in the pre-terminal axons TH is localized in dense grannules and on the rim of vesicles (Pickel et al., 1976). Expression of TH is tightly regulated at the transcriptional, translational and post-translational level (Iwata et al., 2000; Tekin et al., 2014), whereas the activity of TH is regulated by feedback inhibition. Besides being a competitive inhibitor of cofactor tetrahydrobiopterin (Fitzpatrick, 1988), dopamine can bind to the ferric iron of the active site of the enzyme thus preventing the binding of the substrate (Andersson et al., 1988). In addition, activation of dopamine D2 autoreceptors inhibits TH activity by reducing its phosphorylation (Lindgren et al., 2001; Tekin et al., 2014). In neutral pH dopamine reacts with oxygen producing toxic quinones (Hastings and Zigmond, 1994) and therefore the rate of dopamine synthesis is tightly regulated to meet the current needs. Produced dopamine is packed to acidic synaptic vesicles by vesicular monoamine transporter 2 (VMAT2) or metabolized to 3,4-dihydroxyphenylacetic acid, which diffuses out of the neurons. The activity of VMAT2 to transport dopamine to nerve terminals regulates the amount of released dopamine (Pothos et al., 2000). Released dopamine is highly diffuse, the diffusion radius of dopamine has been estimated to be 8 µm in the striatum (Rice and Cragg, 2008). This enables released dopamine to reach large numbers of pre- and postsynaptic, as well as extrasynaptic receptors. After release dopamine is taken up by dopamine transporter (DAT) which is primarily localized outside synapses, along with the dopaminergic fibers (Nirenberg et al., 1996).

In the mammalian midbrain, the dopaminergic cell bodies lie in the substantia nigra pars compacta (SNpc), ventral tegmental area (VTA) and retrorubral field. Cell bodies in the SNpc project mainly to the dorsal striatum (caudate putamen) forming the so-called nigrostriatal pathway. A small fraction of SNpc localized cell bodies innervates cortical and limbic areas. Dopaminergic cell bodies located in VTA project to the nucleus accumbens in the ventral striatum, amygdala, hippocampus, septum and olfactory tubercle, forming the mesolimbic pathway involved in reward and aversion-related cognition, and to the prefrontal cortex forming the mesocortical pathway, involved in executive functions. The tuberoinfundibular pathway comprises of cell bodies located in the hypothalamus projecting to the pituitary gland, regulating endocrine functions. In addition, cell bodies located in the retrorubral field, the dorsal and caudal extension of SNpc, project to striatal, limbic, and cortical areas (Dahlström and Fuxe, 1964; Ungerstedt, 1971a; Björklund and Dunnett, 2007).

The dopaminergic nigrostriatal pathway is of importance for the functionality of basal ganglia, comprising of striatum, both the internal and external part of globus pallidus (GPi and GPe, respectively), subthalamic nucleus (STN), SNpc and substantia nigra pars reticulata (SNpr), and the intralaminar nuclei of thalamus (figure 1). The basal ganglia balances between executing the wanted movement patterns and inhibiting the unwanted, interfering movements. A key player in the function of the basal ganglia is the striatum, which receives glutamatergic input from the cortex and dopaminergic input from the SNpc, modulating the responsiveness of striatal γ-aminobutyric acid (GABA) releasing medium spiny neurons to the cortical input. The major output from the striatum is via GABAergic medium spiny neurons of the direct and indirect pathways. In the direct pathway, dopamine receptor D1, dynorphin, and substance P expressing GABAergic inhibitory projections reach GPi and SNpr (Gerfen and Young, 1988; Le Moine and Bloch, 1995; DeLong and Wichmann, 2007; Surmeier et al., 2014). This further reduces GABAergic transmission in thalamus, allowing glutamatergic transmission in cortex and facilitating wanted movement patterns ("go"). In contrast in the indirect pathway, the striatal inhibitory dopamine receptor D2 and endogenous opioid peptide enkephalin expressing GABAergic projections (Gerfen and Young, 1988; Le Moine and Bloch, 1995) to GPe are activated, decreasing the inhibition of STN and further activation of GPi and SNpr via glutamatergic projections (DeLong and Wichmann, 2007; Surmeier et al., 2014). Thus, the indirect pathway suppresses the interfering unwanted movement patterns by inhibiting thalamus ("no go"). The balance between the direct and indirect pathways determines whether the movement patterns are executed (DeLong and Wichmann, 2007; Surmeier et al., 2014). When the midbrain dopamine signaling is compromised due to degeneration of dopaminergic cell bodies in the SNpc, the delicate balance between the direct and indirect pathway is disturbed, shifting the balance towards the indirect pathway. The activity of GPe is decreased, leading to increased firing of STN glutamatergic neurons and further inhibition of glutamatergic thalamocortical neurons. This basal ganglia dysfunction and abnormal cortical activation pattern are manifested as motor symptoms of PD (Surmeier et al., 2014).

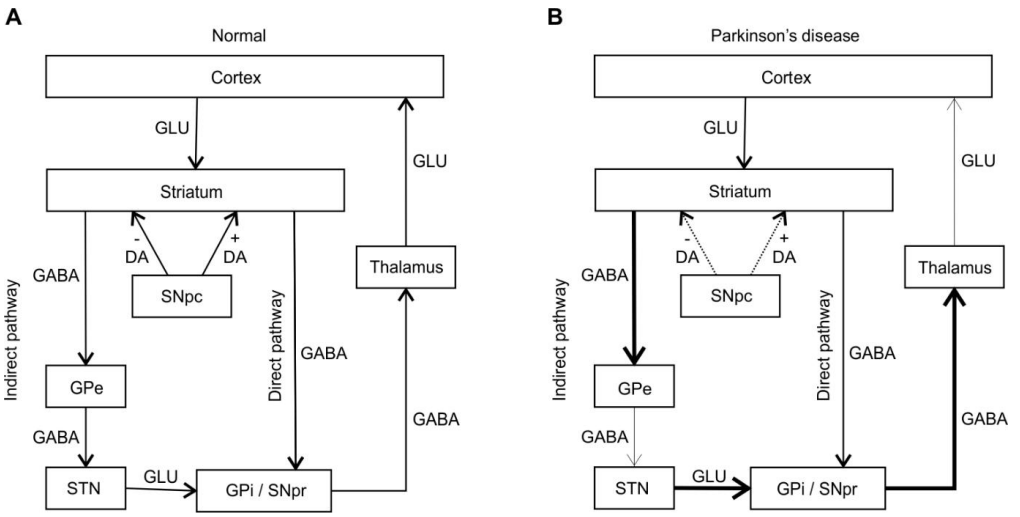

Figure 1. Simplified schematic organization of basal ganglia circuits in A) normal and in B) PD brain (according to DeLong and Wichmann, 2007; Surmeier et al., 2014). In PD, the balance between indirect and direct pathways is impaired due to a decrease in striatal dopamine. DA, dopamine; GABA, γ-aminobutyric acid; GLU, glutamate; GPe, globus pallidus externa; GPi, globus pallidus interna, SNpc, substantia nigra pars compacta; SNpr substantia nigra pars reticulate; STN, subthalamic nucleus.

2.1.2 Parkinson's disease

PD has been estimated to affect 7-8 million individuals worldwide with prevalence of about 1% in population over 60 years old, being the second most common neurodegenerative disease (de Lau and Breteler, 2006; Lindholm et al., 2016). In Finland, there are about 15 000 PD patients (Finnish Parkinson Foundation, www.parkinsonsaatio.fi/parkinsonin-tauti, 14.7.2017). In light of current evidence, the biggest risk factor for PD is age, it is a rare disease in the population of under 50 years old, but the prevalence increases in the older population, reaching 4% in the population of 85 years old and older. Incidence of the disease (cases per 100 000 person-years) increases sharply after 60 years (de Lau and Breteler, 2006). The clinical phenotype of PD is characterized by different motor manifestations, such as bradykinesia, muscle rigidity, and resting tremor, which are most probably caused by impairment of basal ganglia circuitry due to decrease in striatal dopamine levels. However, before the onset of motor symptoms patients have nonmotor symptoms, for instance, hyposmia, depression, cognitive problems, and sleep disorders, and the origin of these symptoms is currently unknown. These nonmotor symptoms can be difficult to recognize and associate with a clinical condition. This further delays the diagnosis, which is determined mainly by the motor symptoms (Postuma et al., 2015; Lindholm et al., 2016). Although the disease has been known for centuries, the first reports about parkinsonian condition are from 300BC from India (Ovallath and Deepa, 2013) and in Western medicine from 1817 (Parkinson, 2002), the exact mechanisms causing this debilitating status are still unclear, hampering the development of disease-modifying therapies for PD.

Most of the PD cases are idiopathic cases, and about 5-10% of the cases are caused by highly penetrant rare mutations, causing so-called monogenic forms of PD (table 1). The first autosomal dominant mutation associated with PD was the SNCA gene encoding α-synuclein (Polymeropoulos et al., 1997). Since that finding, several other pathogenic SNCA missense and structural (i.e. multiplications) mutations have been linked to PD as well as dominant mutations in other genes (reviewed in Lill, 2016). A common feature in the gene mutation-linked PD cases is early onset: mean age of onset is 39 years for parkin, PTEN-induced kinase 1 (PINK1), or DJ-1 homozygous autosomal recessive mutation carriers (Kilarski et al., 2012). In both parkin and DJ-1 genes, several different missense, as well as structural mutations, have been associated with PD (Kitada et al., 1998; Bonifati et al., 2003; Lill, 2016). Although most PD cases are a combination of genetics, epigenetics, environmental and lifestyle factors and no clear phenotype has been connected to a specific mutation (Lill, 2016), linking of specific genes to PD has shed some light on the potential mechanisms of PD pathology.

Table 1. List of genes associated with PD according to OMIM (Online Mendelian Inheritance in Man database, www.omim.org, Parkinson's disease PS168600).

Locus	Protein	Function	Inheritance	Onset
PARK1	α-synuclein	Presynaptic protein	AD	Mid to late adulthood
PARK2	Parkin	E3 ubiquitin ligase	AR	Juvenile
PARK3	Unknown	Unknown	AD	
PARK4	α-synuclein (triplication)	Presynaptic protein	AD	Early
PARK5	UCHL-1	Ubiquitin C-terminal hydrolase	AD	
PARK6	PINK1	Serine/threonine kinase	AR	Early
PARK7	DJ-1	Chaperone, protease, redox sensor, antioxidant scavenger	AR	Early
PARK8	LRRK2	Kinase	AD	Early
PARK9	ATP13A2	ATPase	AR	Juvenile
PARK10	Unknown	Unkown	Unconfirmed	
PARK11	GIGYF2	Insulin-like growth factor signaling	AD	
PARK12	Unknown	Unknown	X-linked/ unknown	
PARK13	HTRA2	Mitochondrial serine peptidase	AD	
PARK14	PLA2G6	Phospholipase	AR	Early
PARK15	FBXO7	E3 ubiquitin ligase component	AR	Early
PARK16	Unknown	Unknown	Unknown	
PARK17	VPS35	Endosome-trans-Golgi trafficking, recycling of membrane-associated proteins	AD	
PARK18	EIF4G1	Initiation of translation	AD	Late adult
PARK19	Auxilin	Clathrin-mediated endocytosis	AR	Early/juvenile
PARK20	SYNJ1	Phosphatase, synaptic vesicle dynamics	AR	Early
PARK21	Unknown	Unknown	AD	Late adult
PARK22	CHCHD2	Transcription factor	AD	
PARK23	VPS13C	Mitochondrial protein	AR	Early
PARK	GBA	Glucosylceramide metabolism (lysosome)	IC, Mu	Late
PARK	ADH1C	Ethanol metabolism	IC, Mu	Late
PARK	TBP	DNA-binding subunit of RNA polymerase II transcription factor	IC, Mu	Late
PARK	Ataxin-2	Epidermal growth factor receptor trafficking	IC, Mu	Late
PARK	MAPT	Microtubule assembly	IC, Mu	Late
PARK	GLUD2	Glutamate metabolism	IC, Mu	Late

AD, autosomal dominant; ADH1C, alcohol dehydrogenase 1C, gamma subunit; AR, autosomal recessive; ATP13A2, ATPase type 13A2; CHCHD2, coiled-coil-helix-coiled-coil-helix domain-containing protein 2; DJ-1, oncogene DJ-1; DNAJC6, DNAJ/Hsp40 homolog, subfamily C, member 6; EIF4G1, eukaryotic translation initiation factor 4 gamma 1; FBOX7, F-box only protein 7; GBA, glucocerebrosidase; GIGYF2, GRB10-interacting GYF protein 2 (PERQ amino acid rich with GYF-domain-containing protein 2); GLUD2, glutamate dehydrogenase 2; HTRA2, HtrA serine peptidase 2; IC, isolated cases, LRRK2, leucine-rich repeat kinase 2; MAPT, microtubule-associated protein tau; Mu, multifactorial; PINK1, PTEN-induced kinase 1; PLA2G6, Phospholipase A2 group 6; SYNJ1, synaptojanin 1; TBP, TATA binding protein; UCHL-1, ubiquitin C-terminal hydrolase; VPS13C, vacuolar protein sorting 13 homolog C.

Pathological hallmarks of the disease are the degeneration of nigral dopaminergic neurons and the presence of Lewy bodies, intracellular protein aggregates consisting of a heterogenous mix of over 90 proteins, such as ubiquitin and the main component, α-synuclein (Kuzuhara et al., 1988; Spillantini et al., 1997; Wakabayashi et al., 2013). However, the complexity of the symptoms observed in patients suggests that also other neuronal pathways, such as noradrenergic, serotonergic, and cholinergic systems, are affected in PD (Parkinson, 2002; Lim et al., 2009; Postuma et al., 2015). For example, the

degeneration of noradrenergic neurons in locus ceruleus and serotonergic neurons in Raphe nuclei with their widely spread projections to many brain areas might pay a role in PD-related mood disorders, sleep disorders, and pain as well as the impairment of the cholinergic system has been associated with gastrointestinal disturbations (Lim et al., 2009).

Braak and colleagues proposed the Lewy pathology to spread from autonomic nerve system to olfactory bulb and lower brain stem in the presymptomatic phase of PD, further to basal midbrain and forebrain in the early symptomatic phase, and in the last, late symptomatic phase to neocortex (Braak et al., 2003). Spreading of Lewy body pathology by cell-to-cell mechanism was further supported by the findings of Kordower and Li (Kordower et al., 2008; Li et al., 2008), who detected α-synuclein positive Lewy bodies in previously grafted neurons in PD patients. Currently, the spreading of the Lewy body pathology is believed to start from the enteric nervous system (Braak et al., 2006), and alterations in the gut microbiome have been suggested to be one of the biomarkers in early PD (Scheperjans et al., 2015). However, the exact mechanisms for the propagation of Lewy body pathology have remained unsolved.

The ascending spreading model of Lewy pathology has also faced some criticism. Evidently, each PD patient is unique and therefore the clinicopathological phenotype of the patients vary. Thus, the relationship between clinical symptoms and the intraneuronal Lewy body pathology (Burke et al., 2008; Jellinger, 2008; Kalaitzakis et al., 2008; Frigerio et al., 2011) as well as the correlation between the Lewy pathology and neuronal death have been questioned (Burke et al., 2008; Surmeier et al., 2017). Moreover, longitudinal data from patients showing the spreading of Lewy body pathology from one brain area to another is still lacking (Surmeier et al., 2017). Recently, Engelender and Isacson (Engelender and Isacson, 2017) proposed a new threshold model for PD. In their model, PD is considered as a global systemic disease and instead of an ascending spreading pattern, the pathology would develop simultaneously in multiple systems (i.e. autonomic, peripheral and central nervous systems). Discrepancies in the functional threshold between the systems would explain why the symptoms arising from different systems appear at different time points.

2.1.3 Dopaminergic neurons in Parkinson's disease

As mentioned above, degeneration of dopaminergic neurons in the SNpc is a pathological hallmark of PD. Several events, such as mitochondrial dysfunction, accumulation of fibrillar α-synuclein forming into Lewy bodies, loss of neurotrophic support and neuroinflammation have been suggested to contribute to the nigral neuronal degeneration in PD (Lill, 2016; Toulorge et al., 2016; Przedborski, 2017). This selective vulnerability of SNpc dopaminergic neurons is believed to emerge from the unique characteristics of the cells, such as their intrinsic pacemaking activity. The ability to release dopamine continuously is suggested to be dependent on L-type calcium channel (with pore-forming Cav 1.3 subunit) (Chan et al., 2007). The cost of this function is the increased oxidative stress due to the calcium influx (Guzman et al., 2010). Another feature of the neurons is the exceptionally wide axonal arborization (Matsuda et al., 2009; Pacelli et al., 2015). Long and unmyelinated axons, such as those of SNpc dopaminergic neurons, have been suggested to be more vulnerable to stress (Braak and Del Tredici, 2004). One SNpc neuron has been estimated to fill on average almost 3% of striatal volume, reaching 2.7% of striatal neurons (Matsuda et al., 2009). In rat, this translates to each striatal neuron to be affected by 95-194 SNpc dopaminergic neurons. This kind of redundancy in the number of neuronal contacts can be thought of as a protective mechanism, as the circuitry can still remain functional after some contacts have been lost (Matsuda et al., 2009). However, the large axonal arborization increases the energy demand of the neuron, and indeed, the axons of SNpc neurons are dense in mitochondria (Pacelli et al., 2015). In addition, the basal rate of the mitochondrial oxidative phosphorylation and production of reactive oxygen species (ROS) in SNpc dopaminergic neurons is high compared to other, less vulnerable dopaminergic neurons (Pacelli et al., 2015). This leads to decreased capacity to respond to cellular stress and increased energy demand.

The neuronal degeneration in PD has been proposed to be a dying-back process, starting from the nerve terminals and propagating along the axon towards the cell bodies (Hornykiewicz, 1998). In fact, it has been estimated that at the time of diagnosis striatal dopamine has decreased about 70-80% (Cheng et al., 2010; de la Fuente-Fernandez, 2013) and about 30% of the nigral cell bodies have degenerated, leaving most of the neurons still viable (Fearnley and Lees, 1991; Cheng et al., 2010). A large pathological study in post-mortem patient samples showed that the striatum (putamen) is almost devoid of TH- and DAT-immunoreactivity five years after PD diagnosis (Kordower et al., 2013). In contrast, in SNpc a small persistent population of dopaminergic neurons is detected decades after the diagnosis (Kordower et al., 2013), supporting the dying back mechanism of the cell degeneration. Indeed, the evidence is pointing at two different degeneration mechanisms: caspase-dependent apoptosis for the cell soma and another, currently unknown mechanism for the preceding selective axon degeneration (Finn et al., 2000; Tagliaferro and Burke, 2016). In a study with mice expressing green fluorescent protein (GFP) in dopaminergic cells, the neurons were revealed to lose their dopaminergic phenotype (i.e. dopaminergic markers) before dying (Cheng et al., 2011). These cells which have lost their dopaminergic phenotype but are still viable provide a target for the disease-modifying intervention. Additionally, the data above suggests that protecting the axons instead of cell bodies might be the key to slow down or even halt the disease progression.

The reduced striatal dopamine is manifested as motor symptoms of PD (Cheng et al., 2010; Lindholm et al., 2016). However, the underlying basal ganglia dysfunction is first counterbalanced by both dopamine-dependent and dopamine-independent mechanisms. The dopamine-dependent mechanisms aim at enhancing the effects of remaining striatal dopamine, for example by increasing dopamine synthesis, slowing down re-uptake of dopamine by decreasing the level of DAT or increasing the level of postsynaptic dopamine receptors. Conversely, dopamine-independent mechanisms aim at reducing the signaling of the indirect pathway of basal ganglia either by reducing corticostriatal input or by reducing the inhibition of GPe (Brotchie and Fitzer-Attas, 2009). This compensation delays the onset of motor symptoms and further, diagnosis of the disease.

2.1.4 Current therapies for Parkinson's disease

The aim of the therapy is to preserve the patient's quality of life by regaining the independence, functionality as well as social competence. Therapies currently available for PD can be divided into three categories: pharmacotherapy, functional neurosurgery, and supportive therapy. Although the pharmacotherapy is most often started with dopamine agonists or monoamine oxidase inhibitors, the cornerstone of pharmacotherapy is levodopa (L-DOPA), the dopamine precursor (Birkmayer and Hornykiewicz, 1961; Oertel and Schulz, 2016). In the beginning, the response to L-DOPA is usually stable despite the fluctuation in the plasma concentration. Nonetheless, during the following years, the response in plasma levels of L-DOPA remains continuous, but the clinical response is decreased and the patients develop dyskinesia (Ahlskog and Muenter, 2001; Oertel and Schulz, 2016). Even with this disadvantage, L-DOPA is still superior as a monotherapy to all other PD medications in its efficacy and short-term side effect profile (Oertel and Schulz, 2016). To increase the availability of L-DOPA in the brain and to minimize peripheral side effects L-DOPA is used in combination with aromatic L-amino decarboxylase inhibitors and catechol-*O*-methyl transferase inhibitor, which inhibit the peripheral metabolism of L-DOPA to dopamine thus reducing the peripheral side effects and enabling the use of lower L-DOPA doses (Oertel and Schulz, 2016).

The second form of therapy is functional neurosurgery, i.e. deep brain stimulation (DBS). Stimulation of the target nucleus, STN, ventral intermediate nucleus, or GPi was developed for the treatment of the motor symptoms of PD. The mechanism of DBS is still unknown, but the electric stimulation is believed to function as a pacemaker, disrupting the disturbed network activity. DBS is often used as a last possibility after L-DOPA response has decreased. Hence, DBS does not replace pharmacotherapy (Oertel and Schulz, 2016). A third type of therapy, supportive therapy, includes various different

functions, such as music therapy, dance, and speech therapy. Physical exercise is known to be beneficial for PD patients (Rafferty et al., 2017) and therefore it is used as a supportive therapy in combination with DBS and pharmacotherapy. The common denominator for all three therapy modes: pharmacotherapy, surgical, and supportive, is that they are all symptomatic, relieving the symptoms of the disease without an effect to the underlying neuronal degeneration (Oertel and Schulz, 2016). Hence, development of new, disease-modifying therapies which would slow down or even stop the progress of the degenerative process, would revolutionize the treatment of PD.

2.2 Animal models of Parkinson's disease

Animal models are essential tools in drug development. Although alternative methods to replace experimental animals are being developed, complex structures, such as the brain, are difficult to model in vitro and therefore animal models are being used and testing in animal models is required for new therapies. However, modeling a disease is not easy. For example, Parkinson's disease is known to have familial forms caused by a genetic mutation, but most of the cases are sporadic, and the factor(s) triggering the disease is not known (de Lau and Breteler, 2006; Duty and Jenner, 2011; Przedborski, 2017). To make things more complicated, animals are not known to have human-equivalent PD. Thus, no perfect animal model has yet been developed, as many of the current animal models fail to exhibit all pathological or behavioral signs of the disease (Duty and Jenner, 2011).

The key term in evaluating animal models is validity. A good animal model should exhibit construct or etiologic validity, meaning similar pathogenesis as the disease, face validity recapitulating the biochemical, behavioral and pathological features of the disease, and predictive validity, ability to distinguish potential new therapies. On that account, a good animal model of PD should have oxidative stress, inflammation, mitochondrial dysfunction and proteasome inhibition (construct validity), decrease in midbrain dopamine levels leading to motor deficits, such as bradykinesia or akinesia, and non-motor symptoms (face validity), and the model should respond well to PD therapies (predictive validity) (Duty and Jenner, 2011; Przedborski, 2017). Besides being a valid model resembling all possible features of the disease, animal models should also be reliable. A reliable animal model is replicable, providing a stable model from animal to animal. In addition to improving the quality of the obtained results with the animal model, high success rate decreases the number of animals needed for the experiments.

2.2.1 Toxin-induced models of Parkinson's disease

6-OHDA model of Parkinson's disease

6-OHDA is a neurotoxin taken up by the cell via monoaminergic transporters DAT and noradrenaline transporter (Luthman et al., 1989; Duty and Jenner, 2011). Since 6-OHDA does not pass blood-brain barrier, it is administered intracranially (Ungerstedt, 1968). Most commonly the model is induced unilaterally, as bilateral injection of 6-OHDA can induce severe welfare problems, such as adipsia and aphagia (Ungerstedt, 1971b). Although cytotoxic mechanisms of 6-OHDA are not fully clear, it seems to have two main modes of action: formation of free oxygen radicals and inhibition of mitochondrial respiratory chain complexes I and IV (Glinka et al., 1997; Mazzio et al., 2004, figure 2). Moreover, 6-OHDA reduces striatal levels of antioxidant enzymes, such as glutathione and superoxide dismutase (Perumal et al., 1992; Kunikowska and Jenner, 2001), and elevates iron level in the SN (Oestreicher et al., 1994). Also, activation of microglia has been observed in the striatum and SN after 6-OHDA injection (Cicchetti et al., 2002) as well as increase in striatal inflammatory markers (Mogi et al., 2000), supporting the construct validity of the 6-OHDA animal model of PD (Duty and Jenner, 2011; Toulorge et al., 2016).

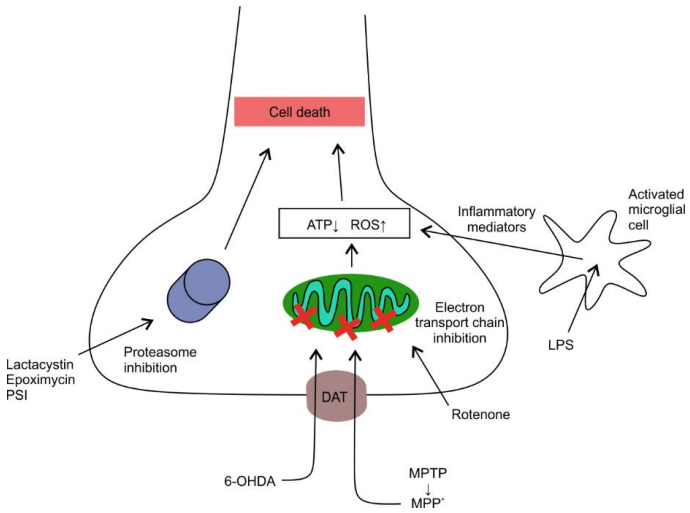

Figure 2. Simplified presentation of mechanisms of toxin-based animal models of PD (adapted from Duty and Jenner, 2011). 6-OHDA, 6-hydroxydopamine; ATP, adenosine triphosphate; DAT, dopamine transporter; LPS, lipopolysaccharide; MPP^+, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PSI, carbobenzoxy-L-isoleucyl-L-gamma-t-butyl-L-glutamyl-L-alanyl-L-leucinal; ROS, reactive oxygen species.

Importantly, the 6-OHDA model also exhibits face validity since administration of 6-OHDA has been shown to induce degeneration of the nigrostriatal pathway (Kirik et al., 1998; Santiago et al., 2014; Toulorge et al., 2016), and increase firing rate of STN (Breit et al., 2007) and basal ganglia output regions entopeduncular nucleus and SNpr as well as increase in glutamate levels (Hutchison et al., 1994; You et al., 1996; Biggs et al., 1997; Breit et al., 2007). However, a significant weakness of the model is the lack of Lewy bodies in the brain, the pathological hallmark of PD (Spillantini et al., 1997; Duty and Jenner, 2011). Parkin-containing aggregates have been reported to be formed in the brain of the animals after 6-OHDA administration (Um et al., 2010), but this finding needs further confirmation.

As discussed earlier, PD is a slowly progressing disease. Conversely, 6-OHDA-induced neurodegeneration evolves faster. The time frame for the cell death depends on the infusion site of the toxin. Infusion of 6-OHDA to medial forebrain bundle (MFB) or SN causes almost complete degeneration of the nigrostriatal pathway in one week (Ungerstedt, 1968; Jeon et al., 1995; Zuch et al., 2000). In contrast, striatal injection of 6-OHDA induces rapid degeneration of the nerve terminals and more slowly progressing partial cell death in SN. Degeneration of the nerve terminals is detectable already 24 hours after 6-OHDA injection and the lesion develops gradually over the following 2-4 weeks (Sauer and Oertel, 1994; Lee et al., 1996; Kirik et al., 1998).

Thus, 6-OHDA model resembles PD in many aspects. It has construct validity, mitochondrial dysfunction, oxidative stress, and inflammation, as well as face validity with degeneration of dopaminergic nigrostriatal pathway and biochemical changes. The model has also limitations, fast cell death and lack of Lewy bodies. Despite its weaknesses, 6-OHDA model has predictive value and it has been used extensively in drug development (e.g. Spencer and Wooten, 1984; Männisto et al., 1992; Wachtel and Abercrombie, 1994).

Behavioral evaluation of the 6-OHDA model of Parkinson's disease

As discussed above, the animal model of PD should have similar pathogenic, biochemical, and behavioral features as PD. Different motor behavior tests are used to assess the development of the lesion in living animals. Most of the tests, such as stepping test, drug-induced rotations or cylinder test are based on the imbalance in the dopamine levels between the hemispheres induced by the unilateral 6-OHDA injection. Probably the most commonly used test is drug-induced rotations (Ungerstedt and Arbuthnott, 1970). Rotations are measured in automated bowls after administration of either a direct dopamine receptor agonist, e.g. apomorphine, or an indirect dopamine agonist, e.g. amphetamine (Ungerstedt and Arbuthnott, 1970; Hudson et al., 1993; Kirik et al., 1998). With apomorphine, the rotational behavior is detected in animals with almost 90% depletion of striatal dopamine (Hudson et al., 1993), making it more suitable for SN or MFB lesions. In contrast, with amphetamine already 40-50% dopamine depletion is observed (Przedborski et al., 1995; Lee et al., 1996; Kirik et al., 1998). However, the amphetamine-induced rotations have poor correlation with nigral cell loss (Lee et al., 1996; Kirik et al., 1998) and might thus fail to detect the functional improvements in lesioned animals. Another drawback of the rotation assay is the possible sensitization of the animals to amphetamine. The animals might rotate in drug-paired environment without drug administration (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Hudson et al., 1994)

Thus, rotational assay might not be the best test to evaluate the loss of dopamine innervation. This has raised the question of how good of a measure the rotational behavior eventually is for testing the efficacy of therapeutic agents (Marin et al., 2006). Therefore use of other tests, such as cylinder test, forelimb placing test or adjusting steps is recommended (Marin et al., 2006; Meredith and Kang, 2006). The advantage of these tests is that they are drug-free, measuring only the animal's normal behavior. In the cylinder test, animals are placed in a plexiglass cylinder and the use of front paws is recorded upon vertical exploration (Schallert et al., 2000). Animals with unilateral lesions use predominantly the ipsilateral front paw. The test has a good correlation only with robust dopamine depletion of 80-90% (Schallert et al., 2000). In contrast to the automated rotation assay, the analysis of cylinder test is subjective and therefore requires a blinded observer to produce reliable results.

MPTP model of Parkinson's disease

Neurodegenerative effects of systemically administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were originally recognized in humans (Langston et al., 1983). This finding led to the development of the first non-human primate model of PD: the MPTP model (Burns et al., 1983). MPTP is highly lipophilic, passing the blood-brain barrier thus enabling systemic administration. MPTP itself is not cytotoxic, but it is metabolized in glia and serotonergic neurons to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPP^+), which exerts cytotoxic effects (Trevor et al., 1988). Similar to 6-OHDA, MPP^+ is taken up by DAT to dopaminergic neurons, where it enters the mitochondria inhibiting mitochondrial complex I, leading to reduced adenosine triphosphate (ATP) production and increased production of ROS (Ramsay and Singer, 1986; Varastet et al., 1994), triggering further apoptotic cell death of the dopaminergic neurons (Duty and Jenner, 2011, figure 2). Since this resembles the mechanisms of PD pathogenesis, the model exhibits construct validity. Interestingly, MPTP is highly toxic in non-human primates and in some mouse strains, but not in rats, for example. The reason for this is not known, but differences in the activity of monoamine oxidase B, which is responsible for converting MPTP to MPP^+ (Zimmer and Geneser, 1987), and in the clearance of the toxic metabolite MPP^+ have been suggested to account for the susceptibility discrepancies (Johannessen et al., 1985).

The MPTP model displays significant face validity (Burns et al., 1983; Duty and Jenner, 2011). Repeated administration of MPTP to non-human primates causes akinesia, bradykinesia and rigidity and postural abnormalities resembling motor symptoms of PD patients (Burns et al., 1983; Jenner et al., 1984). Motor disturbances are also reported in MPTP-treated mice (Fornai et al., 2005; Viaro et al., 2010).

Importantly, also the non-motor symptoms of PD, such as excessive salivation and sleep disturbances, have been observed in MPTP-treated primates (Burns et al., 1983; Jenner et al., 1984; Barraud et al., 2009). MPTP administration induces loss of nigral dopamine cells, decrease in striatal dopamine (Varastet et al., 1994; Fornai et al., 2005) and increased extracellular glutamate (Meredith et al., 2009) together with the elevation of inflammatory markers in the striatum and SN (Kurkowska-Jastrzebska et al., 1999). Non-human primate MPTP model lacks the pathological hallmark of PD, the Lewy bodies (Halliday et al., 2009). In mice, acute and intermittent administration of MPTP did not induce formation of intracellular Lewy body-like inclusions, unlike the long-term continuous infusion of MPTP (Fornai et al., 2005; Shimoji et al., 2005). In addition to construct and face validity, MPTP model has been shown to have predictive validity for assessing symptomatic treatments. For example L-DOPA reversed MPTP-induced hypoactivity (Fredriksson et al., 1990) and catechol-*O*-methyl transferase inhibitor and monoamine oxidase B inhibitor potentiated the effects L-DOPA in MPTP-treated mice (Fredriksson and Archer, 1995). In non-human primate MPTP the predictive value is even better (Close et al., 1990; Jenner, 2003; Duty and Jenner, 2011).

The MPTP mouse model has several advantages over 6-OHDA rat model of PD. Whereas MPTP is administered systemically and induces bilateral degeneration of neurons, successful administration of 6-OHDA requires skillful stereotaxic surgery as the toxin is administered intracranially and most commonly unilaterally. The effects of MPTP administration depend on several factors, such as gender, weight, and strain of the animals (Duty and Jenner, 2011). Moreover, high mortality rates following acute bolus injection of MPTP have been reported (Ferger et al., 2000), though this can be avoided by refining the administration paradigm.

Proteasome inhibition model of Parkinson's disease

One common problem in the toxin models of PD is the lack of Lewy bodies, which are the pathological hallmarks of the disease, reducing the face validity of the animal models. Association of the ubiquitin-proteasome system dysfunctions with PD (Kitada et al., 1998; Leroy et al., 1998; McNaught and Jenner, 2001) inspired the development of proteasome inhibition model of PD. Infusion of proteasome inhibitors, such as lactacystin, to SN has been shown to induce the formation of intracellular proteinaceous α-synuclein-immunoreactive inclusion bodies in rodents (Lorenc-Koci et al., 2011; Pienaar et al., 2015). Moreover, intranigral administration of lactacystin induces degeneration of nigral dopaminergic cell bodies, reduces striatal dopamine and causes motor impairments (McNaught et al., 2002; Lorenc-Koci et al., 2011; Pienaar et al., 2015). The toxic effect however might not be restricted to dopaminergic neurons (Bentea et al., 2017). Microglial activation was also observed around lactacystin-injected SN (Pienaar et al., 2015), supporting the face validity of the model. However, the effects seem to be dependent on the dose and injection site, since such biochemical changes were not detected after intrastriatal infusion of lactacystin (Lorenc-Koci et al., 2011), raising concerns about the replicability of the model (Duty and Jenner, 2011). Another disadvantage of the lactacystin model is that the inhibitors are infused intracranially, thus requiring the stereotaxic surgery similar to 6-OHDA model. Interestingly, dopamine agonist pramipexole (Li et al., 2010) and monoamine oxidase B inhibitors rasagiline and selegiline (Zhu et al., 2008), all currently used in pharmacotherapy of PD, have neuroprotective effects in the proteasome inhibition model of PD. However, no clear disease-modifying effects were observed in clinical trials (Pålhagen et al., 2006; Olanow et al., 2009; Schapira et al., 2013). Yet the model has some predictive validity since both apomorphine and L-DOPA have been shown to have beneficial effects in lactacystin-treated animals (McNaught et al., 2002; Konieczny et al., 2014). In addition to lactacystin, repeated systemic administration of other proteasome inhibitors epoximycin and PSI (carbobenzoxy-L-isoleucyl-L-gamma-t-butyl-L-glutamyl-L-alanyl-L-leucinal) have also been shown to induce degeneration of the nigrostriatal pathway manifested as motor impairments in rats (McNaught et al., 2004), although replication problems have been reported (Kordower et al., 2006b). The replication problems may be the reason why the proteasome inhibition models are not more widely used.

Inflammatory model of Parkinson's disease

Microglial activation has been observed in the brain of PD patients (McGeer et al., 1988; Ouchi et al., 2005; Gerhard et al., 2006). In mice and rats lipopolysaccharide (LPS) from gram-negative bacteria is a potent stimulator of microglia and astrocytes of the central nervous system. Injection of LPS to SN induces rapid activation of microglia, upregulation of proinflammatory cytokines, and generation of ROS (Castano et al., 1998; Arai et al., 2004). Progressive and stable loss of nigral dopaminergic neurons is observed one week after LPS infusion (Castano et al., 1998; Arai et al., 2004), at the same time as animals start to manifest motor impairments (Hunter et al., 2009). However, the LPS model suffers from the same problem as many other toxin models of PD, lack of formation of Lewy bodies (Dutta et al., 2008). Moreover, the predictive value of the model is uncertain as there are no studies available testing the effects of currently used PD medication. Since LPS provides a model for the neuroinflammation-mediated degeneration of dopaminergic neurons, the model has been suggested to have potential in studies regarding the disease progression and in screening for novel diagnostic biomarkers (Dutta et al., 2008).

2.2.2 Animal models based on genetic studies

Autosomal dominant mutations

Physiologically soluble α-synuclein occurs as a tetramer (Bartels et al., 2011), regulating presynaptic vesicle release (Burre et al., 2010). However, in PD fibrillar α-synuclein accumulates forming insoluble intracellular Lewy bodies (Spillantini et al., 1997; Zhou et al., 2011). Several different α-synuclein transgenic mouse lines expressing wildtype (WT) α-synuclein or mutated α-synuclein (for example substitutions A53T and A30P) have been created. The phenotypes of these mouse lines vary a lot, depending on the promoter under which the transgene is expressed, the level of transgene expression, and the age of used animals (Blesa and Przedborski, 2014). In general, many mouse lines develop mild motor alterations without degeneration of nigral dopaminergic neurons. The animals have intracellular α-synuclein and ubiquitin-immunoreactive protein inclusions in the dopaminergic neurons, but these inclusions lack the fibrillar components characteristic to Lewy bodies (Masliah et al., 2000; Paumier et al., 2013; Blesa and Przedborski, 2014). Nevertheless, α-synuclein transgenic mouse lines with mild midbrain dopaminergic neuron loss and motor impairments have also been reported (Lin et al., 2012; Janezic et al., 2013). Besides transgenic animals, overexpression of α-synuclein has been targeted to nigral neurons with different viral vectors (Kirik et al., 2002; Lo Bianco et al., 2002; Oliveras-Salva et al., 2013). The animals exhibit both nigral cell loss and striatal denervation, decreased dopamine level in striatum, and motor impairments. Although intracellular α-synuclein-immunoreactive inclusions have been observed along the nigrostriatal pathway in these animals, these inclusions are ubiquitin-negative and therefore do not recapitulate the phenotype of Lewy bodies (Kirik et al., 2002; Lo Bianco et al., 2002; Oliveras-Salva et al., 2013). Recently the overexpression of commonly used control protein GFP was reported to cause loss of nigral dopaminergic neurons in rats, suggesting that the effects of α-synuclein overexpression might not be α-synuclein-specific (Landeck et al., 2017). Moreover, the use of exogenous promoter might fail to mimic the spatiotemporal expression pattern of α-synuclein in PD, questioning the relevance of α-synuclein overexpressing animals as PD models (Visanji et al., 2016).

In the newly introduced preform fibril model, the preformed α-synuclein fibrils seed the aggregation of endogenous α-synuclein, inducing progressive Lewy body-like pathology with nigral cell loss and motor impairments (Luk et al., 2012). This model might serve as a tool to study the mechanism of formation and cell-to-cell propagation of the fibrillary α-synuclein aggregates. However, there are no evidence that exogenous factor is needed to initiate the spreading of the α-synuclein aggregates (Visanji et al., 2016). Hence, instead of being models for PD, animals overexpressing WT or mutant α-synuclein, or seeded with preformed α-synuclein fibrils can serve as tools to study the α-synuclein

interactome and to screen new therapeutic strategies to inhibit the aggregation of α-synuclein and formation of Lewy bodies (Blesa and Przedborski, 2014; Visanji et al., 2016).

Autosomal recessive mutations

Mutations in parkin and PINK1 genes, which are involved in mitochondrial quality control, are associated with familial forms of PD (table 1, Kitada et al., 1998; Valente et al., 2004). Reduction in mitochondrial membrane potential induces PINK1 to accumulate to mitochondria. Additionally, PINK1 recruits cytosolic parkin to relocate to mitochondria and activates the ubiquitin ligase, initiating the autophagic degradation of damaged mitochondria (Matsuda et al., 2010). A recent report revealed PINK1, parkin, and α-synuclein to act cooperatively in mitochondrial dynamics in mild stress situations. Instead of initiating mitophagy, parkin and α-synuclein interact to induce PINK1-dependent mitochondrial fusion (Norris et al., 2015).

The parkin gene is comprised of multiple exons and different parkin knockout mouse lines have been generated by a deletion in different exons (Blesa and Przedborski, 2014). The mice display some motor deficits and impaired dopamine release as well as reduced DAT levels, but no loss of dopaminergic neurons in SNpc (Itier et al., 2003). Parkin knockout rats did not show any phenotype (Dave et al., 2014). However, bacterial artificial chromosome transgenic mice expressing truncated mutant parkin protein do exhibit an age-dependent loss of both nigral dopaminergic neurons and striatal nerve terminals, followed by reduced dopamine levels in the striatum and motor deficits (Lu et al., 2009). These animals also have an age-dependent accumulation of endogenous proteinase-resistant α-synuclein resembling Lewy body phenotype detected in PD patients (Neumann et al., 2004). Interestingly, transgenic mice overexpressing parkin are less susceptible to MPTP (Bian et al., 2012) and methamphetamine cytotoxicity than WT mice (Liu et al., 2013). In rats, viral vector-mediated parkin overexpression attenuates the toxic effects of both WT and mutant α-synuclein in the nigrostriatal pathway (Lo Bianco et al., 2004; Yamada et al., 2005; Bian et al., 2012). The effects might be dose-dependent, as the overexpression of parkin has been reported to induce degeneration of dopaminergic cells in rats (Van Rompuy et al., 2014). Nonetheless, parkin knockout mice are not the optimal model of PD, since most of the transgenic mouse lines lack the cell degeneration, which is one of the hallmarks of the disease. Yet, parkin KO mice might serve as a model for preclinical PD providing insights into presymptomatic aspects of PD (Itier et al., 2003).

PINK1 knockout mice have an increased number of larger mitochondria in striatum and impairment in the mitochondrial electron transport chain activity, especially complex I, resulting in mitochondrial depolarization. Hence, the mice are more susceptible to oxidative stress and apoptosis (Gautier et al., 2008; Morais et al., 2009). PINK1 deletion has also been reported to cause an age-dependent reduction locomotor activity and brain dopamine content without a decrease in striatal or nigral TH-immunoreactivity (Gispert et al., 2009). In contrast, PINK1 knockout rats develop age-dependent motor impairment and significant, 25% loss of nigral TH-immunoreactive cells at the age of six months and over 50% loss of cells at the age of eight months (Dave et al., 2014).

DJ-1 is a multifunctional protein, including protease activity, transcriptional regulator and most importantly, antioxidant scavenger and redox sensor maintaining the mitochondrial homeostasis (Jiang et al., 2016). Pathogenic mutations in the DJ-1 gene cause recessive, early-onset parkinsonism (Bonifati et al., 2003). DJ-1 protects cells against ROS by self-oxidation at a specific cysteine residue (C106) (Kinumi et al., 2004). Although WT DJ-1 is localized in cytosol, pathogenic forms L166P and M26I that are localized in mitochondria sensitize cells to oxidative stress-induced cell death (Bonifati et al., 2003; Ren et al., 2012). Moreover, in cells overexpressing PD-associated mutant forms of DJ-1, the mitochondria were fragmented and the cells were more prone to ROS-induced oxidative stress. Thus, DJ-1 plays a role in mitochondrial dynamics and protects the cells from oxidative stress (Wang et al., 2012). DJ-1 knockout mice have reduced striatal dopamine release and decreased locomotor activity,

but no degeneration of nigral dopaminergic neurons or α-synuclein positive inclusion bodies (Goldberg et al., 2005). However, when DJ-1 knockout mice were backcrossed with C57BL/6 mice, marked early-onset loss of nigral TH-immunoreactive cells was observed with mild motor impairment (Rousseaux et al., 2012). On the other hand, the DJ-1 knockout rat has been reported to develop motor impairments and loss of nigral TH-immunoreactive cells without any loss of striatal TH-immunoreactivity (Dave et al., 2014). Mice lacking DJ-1 expression are more susceptible to MPTP cytotoxicity, but this can be reversed by adenoviral delivery of DJ-1 (Kim et al., 2005). In line with this, viral vector-mediated overexpression of DJ-1 in the nigral cells of the WT C57Bl/6 mice provides protection against MPTP (Paterna et al., 2007).

MitoPark mice have a disrupted mitochondrial transcription factor A gene, causing reduced mitochondrial DNA expression in midbrain dopaminergic neurons and further, dysfunctional mitochondrial respiratory chain (Ekstrand et al., 2007). The mice display a parkinsonian phenotype with adult-onset slowly progressive motor impairments, such as reduced locomotor activity and rearing behavior, as well as impairment in the retrograde transport leading to degeneration of the nigrostriatal pathway, with loss of striatal innervation and TH-immunoreactive neurons in SN. However, the intracellular protein aggregates observed in dopaminergic neurons were not α-synuclein-immunoreactive (Ekstrand et al., 2007).

In general, the relevance of transgenic animals as models of PD can be questioned. Most of the PD cases are sporadic, under 10% of the cases are due to genetic background (Lill, 2016). The genetic mouse models fail to recapitulate the hallmarks of PD, degeneration of nigrostriatal circuitry and formation of α-synuclein-positive Lewy bodies. The phenotype of each mouse model seems to be very variable. Some of the genes, such as parkin, have several exons and a knockout mouse line can be created by deleting one of these exons. Thus, it seems like the phenotype of the mice might depend on the location of the mutation (Blesa and Przedborski, 2014) and the genetic background of the mouse line (Rousseaux et al., 2012). In many of the cases, the neurochemical and histological changes might be age-dependent and the discrepancies between different studies can be explained just by the age differences (Sanchez et al., 2014; Visanji et al., 2016). Yet the models can provide some insights to preclinical, early abnormalities in the nigrostriatal pathway caused by these mutations and consequently, pathogenic mechanisms leading to cell death. They might also be used as a tool to study compensatory mechanisms. Nevertheless, many of the knockout mouse lines are more susceptible to toxin-induced cell degeneration (Kim et al., 2005; Haque et al., 2012; Karuppagounder et al., 2016), and therefore the combination of genetic model and a toxin model might be more relevant model of PD, combining different aspects of PD (Visanji et al., 2016).

2.3 Targets for disease-modifying therapy in Parkinson's disease

Post-mortem analysis of the PD brain and the genetic studies have shown how wide the etiology of PD is and therefore clinical phenotype of the patients also varies. Studies are pointing at many dysregulated pathways including protein degradation, mitochondrial function, and neurotrophic signaling, contributing to the impairment in neuronal homeostasis resulting in cell death (Lill, 2016; Toulorge et al., 2016; Przedborski, 2017, figure 3). Understanding the pathogenesis of PD is the key to developing new disease-modifying therapies.

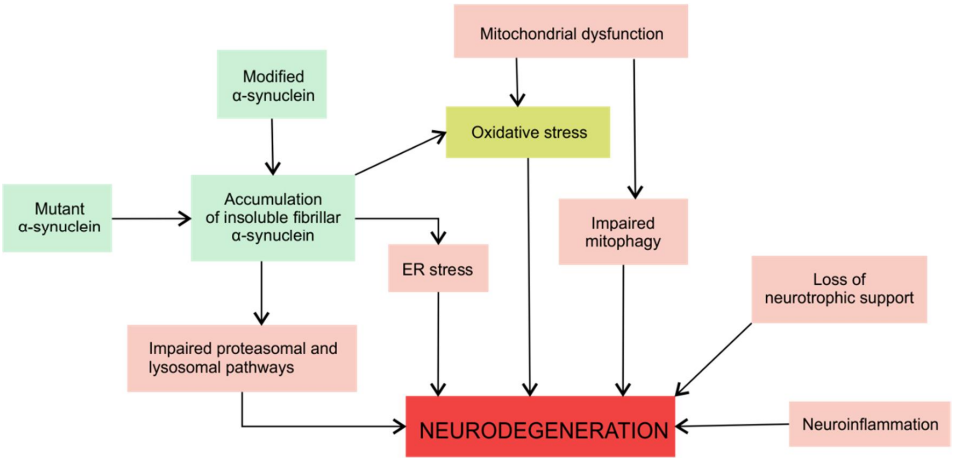

Figure 3. Suggested pathways leading to neuronal degeneration in PD (modified from Przedborski, 2017).

2.3.1 Prevention of formation and spreading of Lewy body pathology

Fibrillar α-synuclein is the main component of intracellular Lewy bodies (Spillantini et al., 1997). Accumulation of insoluble fibrillar α-synuclein has been associated with disturbances in several cellular pathways, inducing for example ER stress (Colla et al., 2012) and oxidative stress (Cremades et al., 2012) leading to cell death. Accumulation of α-synuclein has been linked to other neurodegenerative diseases besides PD (Wong and Krainc, 2017). Formation of α-synuclein aggregates (Cremades et al., 2012) and the spreading of Lewy body pathology in the brain is a slow process (Braak et al., 2003), and therefore prevention of Lewy body formation could serve as a strategy for disease-modifying therapy in PD. Moreover, as discussed in section 2.2.2., several α-synuclein overexpression models have been developed to study the effects of accumulating α-synuclein.

Since the fibrillar form of α-synuclein seems to play the key role in the formation of Lewy bodies, one plausible strategy to reduce Lewy body pathology is to inhibit the aggregation of fibrillar α-synuclein. Several small molecules, such as porphyrin phthalocyanine tetrasulfonate, have been shown to stabilize the physiological forms of α-synuclein, inhibiting formation of fibrillar protein (Fonseca-Ornelas et al., 2014; Schneeberger et al., 2016). Interestingly, intracellular dopamine has been shown to modulate α-synuclein conformation by inhibiting the fibrillization (Conway et al., 2001) and promoting the formation of physiological α-synuclein oligomers (Mazzulli et al., 2006). Thus, reduced intracellular dopamine might lead to formation of fibrillar α-synuclein aggregates. This might further contribute to the selective vulnerability of SNpc neurons in PD.

Another implemented strategy for inhibiting the aggregation of α-synuclein is passive immunization. A monoclonal antibody against α-synuclein fibrils decreased the amount of both soluble and vesicle-bound fibrils in the spinal cord of A30P α-syn-transgenic mice (Lindström et al., 2014). Interestingly, antibody treatment also had beneficial effects on the motor impairments of the animals. Furthermore, passive immunization can attenuate the propagation of α-synuclein-immunoreactive Lewy body-like pathology (Tran et al., 2014). Masliah and colleagues (Masliah et al., 2005) established active immunization, i.e. vaccination, to be efficient in clearance of α-synuclein aggregates in mice expressing human α-synuclein. The formed antibodies recognized abnormal α-synuclein and promoted the degradation of the α-synuclein-immunoreactive protein inclusions via the lysosomal pathway. However, a potential pitfall in the immunization approach is the risk of inducing an α-synuclein-specific autoimmune response (Schneeberger et al., 2016). Therefore vaccines using small peptides mimicking

an epitope of the native α-synuclein molecule have been generated. In phase I clinical trials this approach was proven to be safe, but the efficacy results have not yet been published (Schneeberger et al., 2016) (clinicaltrials.goc Identifiers NCT02267434, NCT02216188, NCT02618941, NCT01885494).

Given that multiplication of the SNCA gene and the following increase in α-synuclein levels is linked to PD in rare cases (Singleton et al., 2003; Chartier-Harlin et al., 2004), strategies to repress α-synuclein expression have been implemented in animal models. Infusion of naked small interfering RNA molecules to hippocampal neurons in vivo reduced the levels of α-synuclein (Lewis et al., 2008), and nigral delivery of viral vectors encoding small hairpin RNA targeted against the SNCA gene also reduced the level of α-synuclein (Sapru et al., 2006; Khodr et al., 2011). Moreover, reduction of α-synuclein levels provided behavioral benefits and protected nigral TH-immunoreactive cells from degeneration in animals overexpressing α-synuclein (Khodr et al., 2011; Khodr et al., 2014). However, at high concentrations, the small hairpin RNA targeted against SNCA had toxic effects on dopaminergic neurons (Khodr et al., 2011). Takahashi and colleagues (Takahashi et al., 2015) tackled this problem by using "expression control RNA interference" with mismatched RNA providing a moderate level of inhibition of α-synuclein expression. This approach was able to restore the α-synuclein level back to normal in the fibroblasts from PD patients and improve the motor impairments of the Drosophila model of PD (Takahashi et al., 2015).

Enhancing protein degradation

Misfolded, damaged and worn-out proteins are removed from the cells by two main protein degradation pathways: ubiquitin-proteasome system (UPS) and autophagy-lysosome pathways (Opattova et al., 2015). Since aggregation of fibrillar α-synuclein is one pathogenic mechanism in PD, enhancing the clearance of the unwanted protein inclusions is an attractive approach for disease-modifying therapy. In normal conditions, α-synuclein is degraded mainly via UPS, but with increased α-synuclein burden the autophagy-lysosome pathway is recruited to help in the degradation process (Ebrahimi-Fakhari et al., 2011).

The UPS is responsible for eliminating unusable, mutant, misfolded, defective, terminally modified and accumulated protein (short-lived proteins) (Opattova et al., 2015). In UPS, misfolded proteins are first tagged with polyubiquitin chain, which is recognized by the proteasome complex (Chau et al., 1989). The tagged proteins are unfolded and degraded to small peptides in the lumen of the complex (Opattova et al., 2015). Interestingly, both α-synuclein and ubiquitin have been observed in Lewy bodies in PD brain (Kuzuhara et al., 1988; Spillantini et al., 1997), and several components of the UPS, such as endogenous UPS activators PA28 and PA700, and proteasome subunits, are dysregulated in parkinsonian brain (Toulorge et al., 2016). In addition, mutations in parkin gene encoding E3 ubiquitin ligase (Kitada et al., 1998) and UCHL-1 gene encoding ubiquitin C-terminal hydrolase L1, which is responsible for recycling the ubiquitin monomers, have been associated with PD (Maraganore et al., 1999), although this link has been challenged (Healy et al., 2006). In a mutant α-synuclein overexpressing mouse model of PD, α-synuclein has been shown to inhibit UPS function (Chen et al., 2006).

Various strategies have been proposed to activate or maintain functional UPS, but only a few approaches have been tested in animal models of PD (Opattova et al., 2015). One of these is the overexpression of ubiquitin ligase parkin to enhance the ubiquitination of misfolded proteins. In non-human primates, striatal co-overexpression of parkin with human α-synuclein attenuated the α-synuclein overexpression induced loss of striatal TH- and DAT-immunoreactivity, thus protecting the dopaminergic phenotype. Co-overexpression with parkin also reduced the neuronal accumulation of α-synuclein (Yasuda et al., 2007). In the rat 6-OHDA model of PD, the viral vector-mediated overexpression of parkin improved the motor impairments but did not show neuroprotective effects on striatal TH-immunoreactive innervation or nigral TH-immunoreactive neurons (Manfredsson et al., 2007). In the lactacystin animal model of PD, where the proteasome function is inhibited, iron

chelators reduced the presence of ubiquitin-positive inclusions and attenuated the loss of nigral neurons (Zhang et al., 2005).

The autophagy-lysosome pathway is responsible for the degradation of damaged or defective intracellular organelles and large protein aggregates (long-lived proteins). Degradable components are transported to acidic lysosomes where a set of hydrolases degrade the components and the building blocks are released back to the cytosol for recycling. Macroautophagy (commonly autophagy) involves initiation and elongation of a double-membraned phagophore (i.e. pre-autophagosomal structure), which sequesters cytoplasmic components until it forms a vesicle called autophagosome. The autophagosome fuses with either an endosome forming an amphisome, or with a lysosome forming an autolysosome. Cytosolic proteins containing a specific degradation signal (KFERQ), which is recognized by Hsc70 chaperone, are translocated to the lysosomal lumen and degraded enzymatically in the chaperone-mediated autophagy pathway. In microautophagy, cytoplasmic components are taken up into the lysosomes (Rivero-Rios et al., 2016).

Accumulating α-synuclein has been suggested to interrupt macroautophagy both in the early phase, inhibiting the formation of autophagosomes (Winslow et al., 2010), and in the late phase, reducing the clearance of autophagosomes (Tanik et al., 2013). In addition, mutant α-synuclein can inhibit the chaperone-mediated autophagy by blocking the lysosomal membrane receptors. This results in reduced degradation of α-synuclein as well as other proteins (Cuervo et al., 2004). Moreover, mutations in several other PD-linked genes, such as LRRK2, ATP12A2, PINK1, and VPS35, have been shown to impair the autophagy-lysosome pathway (Rivero-Rios et al., 2016) and many other proteins involved in the pathway are dysregulated in the parkinsonian brain (Toulorge et al., 2016).

Thus, the dysfunctional autophagy-lysosome pathway results in accumulation of misfolded proteins as well as aggregation of proteins and damaged cell organelles leading to cell death (Cuervo et al., 2004). A variety of pharmacological autophagy-lysosome pathway inducers have been tested in PD models. Rapamycin is an allosteric inhibitor of mTOR (mammalian target of rapamycin) kinase, which is involved in the regulation of the autophagy-lysosome pathway. Intracranial infusion of rapamycin enhanced the autophagy-lysosome pathway and decreased accumulation of α-synuclein in transgenic mice expressing human α-synuclein (Crews et al., 2010). In contrast, non-reducing saccharide trehalose induces autophagy via a mTOR-independent mechanism. However, trehalose and rapamycin have an additive effect in enhancing the clearance of mutant α-synuclein via the autophagy-lysosome pathway in transgenic mouse lines (Sarkar et al., 2007). Interestingly, trehalose alone has beneficial effects in the MPTP mouse model PD. Pretreatment with trehalose protected striatal dopaminergic neurites and nigral cell bodies from cytotoxicity of chronic MPTP administration (Sarkar et al., 2014). The problem with trehalose is that it does not penetrate blood-brain barrier, and in the gut, it is gestated to glucose (Rivero-Rios et al., 2016). Xilouri and coworkers used a gene therapy approach to enhance the chaperone-mediated autophagy (Xilouri et al., 2013). Co-overexpression of Lamp2a, a rate-limiting enzyme of the chaperone-mediated autophagy pathway, with human WT α-synuclein in nigral dopaminergic neurons reduced the amount of phosphorylated α-synuclein as well as formation of α-synuclein-immunoreactive Lewy body-like inclusions in the ventral midbrain. Furthermore, enhancement of chaperone-mediated autophagy by Lamp2a overexpression protected the nigral dopaminergic cell bodies and striatal nerve terminals from α-synuclein-induced degeneration (Xilouri et al., 2013).

Decressac and colleagues, in contrast, enhanced autophagy by increasing lysosomal capacity (Decressac et al., 2013). Transcription factor EB (TFEB) regulates the expression of genes involved in lysosomal biogenesis and autophagosome formation. Overexpression of α-synuclein impairs the function of TFEB by sequestering it to the intracellular inclusion bodies. In rats, overexpression of TFEB and beclin1 impeded the motor deficits induced by overexpression of α-synuclein in SN. In addition, TFEB protected the nigral dopaminergic neurons and striatal nerve terminals and maintained the striatal dopamine level (Decressac et al., 2013).

2.3.2 Unfolded protein response

Accumulation of unfolded proteins in the ER, which maintains protein homeostasis, induces ER stress. ER stress triggers the unfolded protein response (UPR) by activating transmembrane sensors inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 (ATF6), and double-stranded RNA-activated protein kinase-like ER kinase (PERK) (figure 4, Mercado et al., 2013). Activation of these sensors leads to activation of transcription factors X-box-binding protein 1, transcriptional activator domain of ATF6, and activating transcription factor 4, regulating the expression of genes involved in protein homeostasis, degradation of misfolded proteins, and autophagy. In chronic ER stress, when UPR pathway is unable to restore homeostasis, apoptosis is triggered (Mercado et al., 2013). In the PD brain, several ER stress markers are upregulated, supporting the role of ER stress as one of the pathogenic mechanisms (Toulorge et al., 2016). Moreover, several studies have shown activation of UPR markers in both toxin and genetic animal models of PD (Mercado et al., 2013).

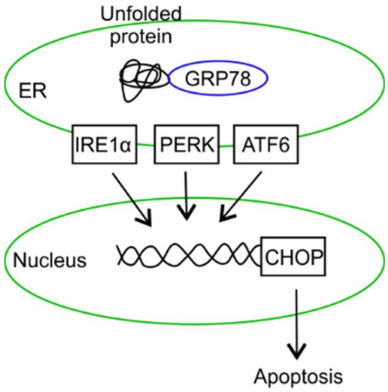

Figure 4. Unfolded protein response. GRP78 recognizes unfolded proteins in the ER triggering the unfolded protein response via IRE1α, ATF6 or PERK Modified from (Mercado et al., 2013). ATF6, activating transcription factor 6; CHOP, CCAAT-enhancer-binding protein homologous protein; ER, endoplasmic reticulum; GRP78, glucose regulated protein 78; IRE1α, inositol-requiring enzyme 1α; PERK, double-stranded RNA-activated protein kinase-like ER kinase.

Thus, enhancing the UPR has been considered as a novel strategy for PD therapy. Indeed, activators of the UPR pathways have neuroprotective effects in animal models of PD. Gorbatyuk and coworkers enhanced the first step of the UPR by overexpressing glucose regulated protein 78 (GRP78), ER resident chaperone recognizing the unfolded proteins in nigral dopaminergic neurons in rats (Gorbatyuk et al., 2012). Co-overexpression of GRP78 with human α-synuclein prevented the degeneration of nigral dopaminergic cells and maintained striatal dopamine concentrations. The beneficial effects of GRP78 overexpression in the rotational asymmetry test correlated with dopamine depletion and loss of TH-immunoreactive cells, supporting therapeutic potential of targeting GRP78 in PD (Gorbatyuk et al., 2012). Moreover, overexpression of GRP78 reduced the expression of CCAAT-enhancer-binding protein homologous protein (CHOP), a proapoptotic protein in the nigral cells (Gorbatyuk et al., 2012). Another strategy is to target only one pathway of the UPR instead of a more general approach. Administration of methoxyflavone derivative tangeretin activates the PERK pathway of the UPR, which resulted in protection of nigral TH-positive neurons from MPTP cytotoxicity (Hashida et al., 2012). Valdes and colleagues, in contrast, enhanced the IRE1α pathway by overexpressing a downstream regulator, transcription factor XPB1 in SNpc in mice. This approach was efficient in protecting nigral dopamine neurons from 6-OHDA toxicity (Valdes et al., 2014). Different compounds have also been tested as pharmacological chaperones (pharmacoperones) to reduce UPR. Pharmacological chaperones are small molecules stabilizing the native conformation of a protein and preventing their misfolding. For example, nicotine suppresses tunicamycin-induced ER stress and the following UPR in mouse ventral midbrain neurons in vitro (Srinivasan et al., 2016).

The role of the UPR is to maintain the homeostasis and cell viability as an adaptive mechanism. Hence, mild ER stress is actually neuroprotective. This hypothesis is supported by results from Mollereau group, showing that predisposition to mild ER stress induced by tunicamycin is neuroprotective against

6-OHDA-induced cytotoxic insult in mice (Fouillet et al., 2012). Tunicamycin pretreatment increased the number of remaining TH-immunoreactive cells in SNpc and antagonized 6-OHDA-induced rotational asymmetry. Additionally, in human α-synuclein expressing Drosophila model of PD, tunicamycin pretreatment improved climbing ability of the flies. In both models, the beneficial effect of ER stress is mediated via an autophagy-dependent mechanism inhibiting apoptosis (Fouillet et al., 2012).

2.3.3 Mitochondria and oxidative stress

Mitochondria are cell organelles responsible for essential cellular functions such as maintaining balance in the energy metabolism by producing ATP, neuronal survival, calcium buffering, and apoptotic signaling. Several studies suggest mitochondria to play a role in PD pathogenesis. Observation of decreased activity of complex I of mitochondrial electron transport chain (figure 5) in SN of the PD patients (Schapira et al., 1989) was followed by detection of high levels of deletions in mitochondrial DNA in SN neurons in PD patients, but also in aged controls (Bender et al., 2006). In vivo and in vitro studies have shown PD-associated genes and mutations, such as α-synuclein point mutations or PINK1 deficiency, to induce mitochondrial dysfunction either by disrupting mitochondrial dynamics or by inhibiting oxidative phosphorylation. Moreover, PD-related protein parkin is involved in mitophagy, degradation of dysfunctional mitochondria via autophagy (Narendra et al., 2008; Hu and Wang, 2016). Indeed, several mitochondrial and oxidative stress associated genes (Zheng et al., 2010) and proteins (Toulorge et al., 2016) are dysregulated in parkinsonian brain. In addition, as discussed earlier, several animal models of PD have defects in mitochondrial function (Duty and Jenner, 2011; Hu and Wang, 2016). Recently, Amano and colleagues suggested the motor symptoms to be an adaptive mechanism to metabolic dysfunction underlying the neuronal vulnerability (Amano et al., 2014). Whether mitochondrial dysfunction is the cause of the disease or if it correlates with disease progression, remains debated (Franco-Iborra et al., 2016). Thus, the bioenergetics imbalance would serve as a plausible therapeutic target in PD.

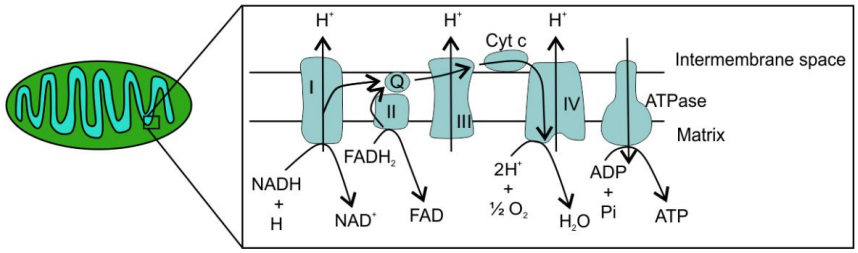

Figure 5. Electrons passing electron transport chain complexes in the inner membrane of mitochondria create proton gradient which is used by ATPase to create ATP. Modified from (Jiang et al., 2016). ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt c, cytochrome c; FAD, Flavin adenine dinucleotide; NAD, nicotine amide adenine dinucleotide; Pi, phosphate; Q, coenzyme Q10.

NADPH oxidase and mitochondrial oxidative phosphorylation are the two major ROS producers in cells. Cellular antioxidants, such as superoxide dismutase, glutathione-S-transferase, and melatonin, take care of the redox homeostasis, protecting cells from oxidative stress. However, when ROS production exceeds the cellular antioxidant activity, oxidative stress induces accumulation of cytotoxic compounds leading to cell death (Jiang et al., 2016). Moreover, dopamine can be oxidized to quinones (Hastings and Zigmond, 1994), which can further modulate the formation of physiological α-synuclein oligomers (Conway et al., 2001; Jiang et al., 2016). Thus, one plausible therapeutic strategy would be to enhance the function of cellular antioxidants. However, the results obtained with administration of antioxidant vitamin E in the MPTP models of PD have been contradictory (Perry et al., 1985; Perry et al., 1987). Coenzyme Q is an electron carrier in the electron transport chain buffering the excessive ROS

generation. Dietary coenzyme Q was shown to be effective in protecting the nigrostriatal tract against MPTP-induced degeneration in mice. Coenzyme Q-treated animals had more striatal dopamine and TH-positive fibers left after MPTP treatment compared to control group (Beal et al., 1998). Positive results in vivo led to clinical trials, but the coenzyme Q-derived mitoquinone did not have an effect on the disease progression (Snow et al., 2010).

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor that regulates expression of many genes involved in the cellular antioxidant response, such as glutathione. In normal conditions, Nrf2 is a cytosolic protein, but it localizes to the nucleus as a response to oxidative stress. In nigral neurons derived from PD patients, Nrf2 is strongly localized to the nucleus (Ramsey et al., 2007). Pharmacological activation of Nrf2 has been shown to be neuroprotective against MPTP (Jazwa et al., 2011), even when the expression is restricted to astrocytes (Chen et al., 2009). Moreover, astrocyte-specific expression of Nrf2 provides neuroprotection in mice overexpressing human mutant α-synuclein (A53T) by reducing the total amount of α-synuclein and phosphorylated α-synuclein by enhancing the degradation of α-synuclein via the autophagy-lysosome pathway (Gan et al., 2012). Interestingly, physical exercise activated the Nrf2 pathway in 6-OHDA lesioned mice (Aguiar et al., 2016).

As discussed earlier, large unmyelinated axonal arbor and the continuous pacemaking activity increase the energy demands of the nigral dopaminergic cells and predisposes the cells to oxidative stress (Chan et al., 2007; Guzman et al., 2010; Pacelli et al., 2015). Thus, antagonizing L-type calcium channels might serve as a disease-modifying therapy. Indeed, use of blood-brain barrier passing dihydropyridine calcium channel blockers as hypertensives has been associated with reduced risk of PD (Becker et al., 2008; Pasternak et al., 2012). In both mouse and nonhuman primate MPTP models, pretreatment with calcium antagonist nimodipine protects nigral dopaminergic cell bodies but not striatal nerve terminals from cytotoxic insult (Kupsch et al., 1995; Kupsch et al., 1996). This discrepancy can be due to different cytotoxicity mechanisms for striatal and nigral effects of MPTP (Kupsch et al., 1995; Kupsch et al., 1996). However, in 6-OHDA treated mice, pretreatment with another calcium channel antagonist isradipine protected both striatal nerve terminals and nigral cell bodies from degeneration (Ilijic et al., 2011). Currently, the efficacy of isradipine in early stage PD patients is being assessed, but the results have not yet been published (clinicaltrials.gov identifier NCT02168842).

One strategy to target mitochondrial dysfunction is to improve mitochondrial biogenesis via a transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), a dominant regulator of mitochondrial function and biogenesis. Targeting of PGC-1α is further supported by the finding that both PGC-1α itself (Eschbach et al., 2015) and a large set of PGC-1α regulated genes are downregulated in the PD brain (Zheng et al., 2010). Additionally, SNpc dopaminergic neurons have a higher level of PGC-1α mRNA than in VTA neurons (Pacelli et al., 2015), demonstrating the sensitivity of dopaminergic neurons to modifications of mitochondrial gene expression and biogenesis (Ciron et al., 2012). α-synuclein, on the other hand, has been shown to repress PGC-1α expression by direct interaction with its promoter (Siddiqui et al., 2012). Downregulation of PGC-1α can further induce aggregation of fibrillar α-synuclein and formation of Lewy body-like inclusions and the toxic effects, whereas elevation of PGC-1α activity reduces the aggregation of fibrillar α-synuclein and attenuates the toxic effects (Eschbach et al., 2015). The SNpc dopaminergic neurons of PGC-1α knockout mice have larger and clustered mitochondria, a phenotype associated with mitochondrial dysfunction and cell death (Ciron et al., 2015). The neurons also display fragmented ER with less mitochondrial contacts compared to WT mice. Studies using PGC-1α knockout mice have shown PGC-1α to protect dopaminergic neurons from cytotoxic insults of MPTP (St-Pierre et al., 2006) and viral vector-mediated overexpression of human α-synuclein (Ciron et al., 2015) by reducing oxidative stress. However, viral vector-mediated overexpression of PGC-1α in rat nigrostriatal system induced degeneration of dopaminergic neurons and consequent decrease in striatal dopamine in high doses (Ciron et al., 2012). Since the long-term overexpression of PGC-1α with viral delivery might be unfavorable, use of small molecules, such as resveratrol might serve as a more controlled approach (Mudo et al., 2012).

Intraperitoneal administration of resveratrol induced the expression of PGC-1α in WT mice and reduced MPTP-induced neurodegeneration (Mudo et al., 2012).

2.3.4 Neuroinflammation

In PD patients, a strong glial reaction has been observed in the SN (McGeer et al., 1988) and a large number of inflammatory-related proteins functioning in different immune response pathways are upregulated in the parkinsonian brain (Toulorge et al., 2016). Indeed, use of non-steroidal anti-inflammatory drugs has been suggested to have weak protective effects against PD (reviewed in McGeer and McGeer, 2008). In early PD patients, a radiotracer study showed clear correlation between microglial activation and dopaminergic terminal loss (Ouchi et al., 2005). However, in later stage PD patients, the microglial activation remains on the same level whereas the dopaminergic terminal loss progresses over time (Gerhard et al., 2006). Consequently, microglial activation has been suggested to initiate an immune response, which is then amplified by astrocytes and this has been hypothesized to enhance the ongoing degeneration (Halliday and Stevens, 2011). In an animal model of PD, induction of inflammatory response by LPS has been shown to cause degeneration of the nigrostriatal pathway (Castano et al., 1998; Arai et al., 2004).

Accumulation of fibrillar α-synuclein and formation of Lewy bodies have been suggested to trigger the immune response in the brain (Reynolds et al., 2008; Van der Perren et al., 2015). Administration of FK506 (tacrolimus), an immunosuppressant used after organ transplantations to decrease the recipient's immune response, attenuated neuroinflammation in rats overexpressing A53T mutant α-synuclein in nigral dopaminergic neurons. This FK506-mediated suppression of both T-lymphocyte pathway and interleukin 2 transcription was able to improve the survival of nigral dopaminergic neurons. However, FK506 did not have an effect on the α-synuclein level (Van der Perren et al., 2015). This observation is in contrast to previously published data, where FK506 did reduce α-synuclein aggregation and formation of Lewy body-like inclusions as well as neuronal cell death in mice overexpressing α-synuclein (Gerard et al., 2010).

However, since many different inflammatory-related proteins are upregulated, suggesting that many different pathways play a role in the inflammatory response of a PD brain, manipulation of a single protein or pathway might not be the optimal strategy to fight inflammation in PD. Instead, the therapeutic agents should have a broader spectrum of action on the inflammatory processes to protect neurons from degeneration (Hirsch et al., 2003). This hypothesis is supported by a study by Yong et al., who showed that a vaccine against childhood tuberculosis has neuroprotective effects in a MPTP mouse model of PD (Yong et al., 2011). Bacille Calmetté-Guerin vaccine preserved striatal DAT and dopamine levels while preventing the MPTP-induced microglial activation in SN. Thus, a peripheral immune response can be beneficial in neuropathological conditions. Together, the results both with FK506 and tuberculosis vaccine suggest that although modulation of the immune response does not attenuate the pathological process, it might alter the environment to be more neurosupportive and hence slow down the progress of the disease.

2.3.5 Augmenting neurotrophic support with GDNF family ligands

Neurotrophic factors play an important role in the neuronal development by regulating neurite branching, synaptogenesis, and maturation of neuronal phenotype, as well as maintenance and survival of neuronal functions and connections of mature neurons (Airaksinen and Saarma, 2002). According to the classical neurotrophic factor theory, small quantities of neurotrophic factors are released from the target tissue, and only the neurons which are able to establish contacts with the target cells will receive enough trophic support to survive (Levi-Montalcini, 1987). However, most likely the situation is more complex in the central nervous system. In the PD brain, several neurotrophic factors are downregulated in the nigrostriatal pathway and the trophic support is missing from the

dopaminergic neurons (Toulorge et al., 2016). Hence, the restoration of the trophic support has been considered as a potential disease-modifying therapy for PD. Classical neurotrophic factors include three protein families: neurotrophins (nerve growth factor, brain-derived neurotrophic factor, NT-3, and NT-4), neurotrophic cytokines (e.g. ciliary neurotrophic factor, interleukin 6, leukemia inhibitory factor, cardiotrophin 1), and GDNF family ligands (GFLs; GDNF, neurturin (NRTN), artemin, and persephin) (Bespalov and Saarma, 2007), which are the focus of this thesis. The fourth family of neurotrophic factors comprises of cerebral dopamine neurotrophic factor (Lindholm et al., 2007) and mesencephalic astrocyte-derived neurotrophic factor (Petrova et al., 2003).

GDNF family ligands

GFLs are distant members of transforming growth factor β-superfamily. All GFLs are functional as disulfide-bonded cysteine knot stabilized homodimers (Butte, 2001) and signal via a two-step mechanism, first uniting two lipid raft-resident glycosylphosphoinositol-anchored GDNF receptor αs (GFRα) and recruiting further two Ret (Rearranged during transfection) receptors. Following the formation of heterohexameric complex and transphosphorylation of tyrosine residues, Ret can initiate intracellular signaling cascades such as mitogen-activated protein kinase and phosphoinositide 3-kinase pathways (Hayashi et al., 2000; Airaksinen and Saarma, 2002; Bespalov and Saarma, 2007; Kramer and Liss, 2015). Hence, GFLs do not bind directly to tyrosine kinase Ret, but instead to coreceptors GFRα1-4. Each GFL has its preferential coreceptor, GDNF binds to GFRα1, NRTN GFRα2, artemin to GFRα3, and persephin to GFRα4, but some cross-talk does occur between GFLs and GFRαs (figure 6, Bespalov and Saarma, 2007; Kramer and Liss, 2015). Besides Ret, GFLs can initiate the signaling cascade via neural cell adhesion molecule (Paratcha et al., 2003) or syndecan-3 (Bespalov et al., 2011).

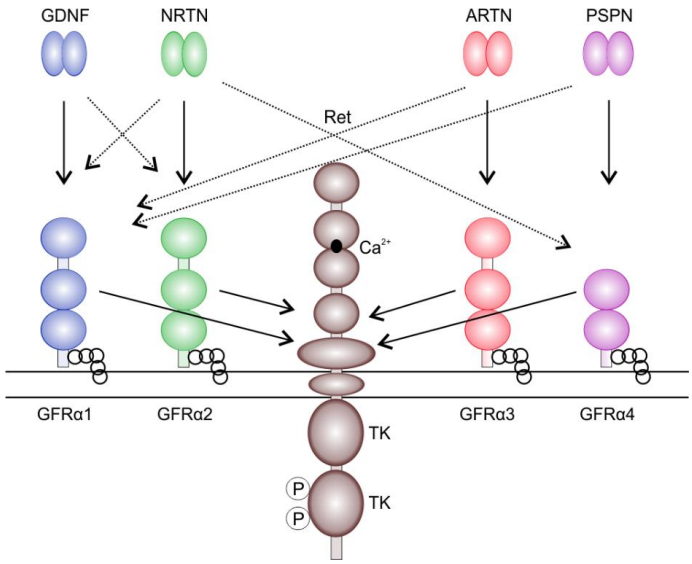

Figure 6. Initiation of GDNF family ligand signaling. Adapted from (Airaksinen and Saarma, 2002; Kramer and Liss, 2015). ARTN, artemin; GDNF, glial cell line-derived neurotrophic factor; GFRα, GDNF receptor α; NRTN, neurturin; PSPN, persephin; Ret, rearranged during transfection; TK, tyrosine kinase.

GFLs have an effect on survival of several neuronal subtypes, including sympathetic, parasympathetic, somatosensory, motor, and dopaminergic neurons (Airaksinen and Saarma, 2002). In addition to neuronal populations, GFLs play a role in non-neuronal processes, for example in kidney development

(Davies et al., 1999; Sariola and Saarma, 2003) and spermatogenesis (Viglietto et al., 2000; Sariola and Saarma, 2003).

Neurobiology of GDNF

Originally, glycosylated disulfide-bonded homodimer GDNF was purified from rat glial cell line B49 (Lin et al., 1993). Despite having supporting effects on several neuronal types and outside the central nervous system (Bespalov and Saarma, 2007), GDNF has caught attention due to its protective effects on dopamine neurons since its discovery (Lin et al., 1993). In midbrain dopaminergic neuron cultures, GDNF promoted survival of dopaminergic neurons but did not have an effect on GABAergic or serotonergic neurons, thus exhibiting neuronal specificity. Furthermore, GDNF increased the morphological differentiation, neurite outgrowth, and the cell body size of dopaminergic neurons (Lin et al., 1993) as well as formation of new axon terminals (Bourque and Trudeau, 2000). These observations led to experiments with animal models of PD, where GDNF also exhibited potential in protecting the dopaminergic neurons from cytotoxic insults (Hoffer et al., 1994; Kirik et al., 2000a).

In general, GDNF mRNA is expressed in higher level in the peripheral tissues, such as kidney, gut, testis, and ovary than in the central nervous system (Suvanto et al., 1996; Trupp et al., 1997; Golden et al., 1999). In the brain GDNF expression is higher in the early postnatal days than in adulthood (Choi-Lundberg and Bohn, 1995). In adult brain the expression of GDNF mRNA is highest in the striatum, nucleus accumbens, septum and thalamus (Trupp et al., 1997; Hidalgo-Figueroa et al., 2012). In striatum, GDNF is mainly expressed in parvalbumin-positive interneurons, which represent only about 0.7% of the striatal neurons (Hidalgo-Figueroa et al., 2012). Small population of cholinergic and somatostatin-positive interneurons express also GDNF mRNA. Interestingly, medium spiny neurons, which receive dopamine innervation, do not express GDNF. However, the expression of GFRα is more restricted than GDNF expression and the difference in the expression patterns is supporting the target-derived paracrine function of GDNF (Choi-Lundberg and Bohn, 1995; Suvanto et al., 1996; Trupp et al., 1997; Golden et al., 1999).

GDNF is produced as a precursor protein, pre-pro-GDNF. The pre-region is proteolytically cleaved off in ER and the pro-region in secretory vesicles (Lin et al., 1993; Lonka-Nevalaita et al., 2010). Considering that the pro-forms of other neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) have been shown to differ in their biological activity from their mature forms (Lee et al., 2001; Gibon et al., 2016), it is surprising that there are only a few reported studies about the biology of pro-GDNF. Pro-region has been suggested to enhance the protein folding and secretion of GDNF (Piccinini et al., 2013), but GDNF is also secreted without its pro-region (Lonka-Nevalaita et al., 2010; Piccinini et al., 2013). Moreover, pro-GDNF has been suggested to be the prevailing form in the brain (Sun et al., 2014), but this observation needs further confirmation.

GDNF has two pro-isoforms generated by alternative splicing of the third exon. The full-length pre-α-pro-GDNF (α-GDNF) localizes in the Golgi-like structures and is secreted constitutively, whereas the shorter pre-β-pro-GDNF (β-GDNF), which has a deletion of 26 amino acids in the pro-region, is located in the vesicles of the regulated secretory pathway and secreted activity-dependently (Lonka-Nevalaita et al., 2010). In contrast, the secretion mechanism of α-GDNF is not known. The mature GDNF produced by both isoforms, however, is similar (Trupp et al., 1995; Lonka-Nevalaita et al., 2010). The isoforms are expressed in parallel in same tissues but in varying proportions (Suter-Crazzolara and Unsicker, 1994; Trupp et al., 1995; Airavaara et al., 2011), e.g. during development β-GDNF is expressed more than α-GDNF (Suter-Crazzolara and Unsicker, 1994). The full-length pro-region of GDNF includes a biologically active 11-mer peptide, dopamine neuron stimulating peptide-11 (DNSP-11 or brain excitatory peptide, BEP) (Immonen et al., 2008; Bradley et al., 2010). In the severe 6-OHDA lesion model of PD, DNSP-11 induced behavioral recovery and increased the dopamine level in the lesioned striatum (Bradley et al., 2010). The effects of DNSP-11 are interesting since α-GDNF is expressed more than β-GDNF in human brain (Airavaara et al., 2011).

GDNF exerts its effects via GFRα1 and Ret (Jing et al., 1996) or neural cell adhesion molecule (Paratcha et al., 2003). Alternatively GDNF can initiate the signaling cascade by binding to syndecan-3 Ret-independently (Bespalov et al., 2011). To terminate the signaling GDNF/GFRα1 complex is internalized through sortilin-related receptor SorLA and directed to the endosomes (Glerup et al., 2013). From the endosomes GFRα1 is recycled to the receptor pool and GDNF transported to lysosomes for degradation.

The importance of GDNF in development is demonstrated in mice lacking GDNF. The homozygous GDNF knockout mice die shortly after birth due to absence of kidneys and enteric neurons (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). The mice have also defects in dorsal ganglion, sympathetic, and nodose neurons, but the dopaminergic system is intact, suggesting that GDNF is not indispensable for the development of dopaminergic neurons (Moore et al., 1996; Sanchez et al., 1996). In contrast, heterozygous GDNF knockout mice are viable, but have defects in spermatogenesis (Meng et al., 2000) and cognition (Gerlai et al., 2001). Whether GDNF is needed for the maintenance of dopaminergic system in the adulthood, is less clear. When GDNF expression was suppressed in adult mice, the animals exhibited reduced locomotor activity as well as pronounced progressive loss of catecholaminergic neurons in SNpc, ventral tegmental area, and locus coeruleus (Pascual et al., 2008). However, these results were challenged recently by Kopra and colleagues, who did not observe changes in the catecholamine system of conditional GDNF knockout mice (Kopra et al., 2015). This discrepancy between the studies might be due to differences in the genetic backgrounds of the animals (discussed in Pascual and Lopez-Barneo, 2015).

Although there has been a major interest in the dopaminotrophic effects of GDNF, the link between PD and GDNF is weak. Chauhan and coworkers reported a significant decrease in GDNF protein expression in SNpc neurons in PD brain compared to healthy controls (Chauhan et al., 2001). However, the study was conducted with immunohistochemistry and therefore must be interpreted cautiously. In another study the GDNF levels were studied with enzyme-linked immunosorbent assay, and no differences in the GDNF levels in caudate, putamen, or SN between PD patients and healthy controls were observed (Mogi et al., 2001).

Neurobiology of NRTN

NRTN was discovered by its ability to enhance the survival of sympathetic neurons in culture (Kotzbauer et al., 1996). The following studies presented the neuroprotective properties of NRTN on midbrain dopaminergic neurons, as NRTN was able to protect dopaminergic neurons as efficiently as GDNF both in vitro and in vivo (Horger et al., 1998). Interestingly, NRTN was found to be more persistent in brain tissue and it has a more long-lasting effect on dopamine turnover in rat striatum than GDNF (Hadaczek et al., 2010). However, the reported lack of the capability to induce axonal sprouting and hypertrophy of dopaminergic neurons (Åkerud et al., 1999) might be due to poor solubility in neutral pH (Rosenblad et al., 1999).

During mouse embryonic development NRTN is highly expressed in peripheral tissues, such as gut, liver, and skin, but also in dorsal root ganglion and motor neurons (Golden et al., 1999). In ventral midbrain, where the NRTN-responsive dopaminergic neurons are located, NRTN is expressed in early embryonic development (until E14). In contrast, NRTN receptor GFRα2 and Ret are expressed in ventral midbrain throughout whole embryonic development period and in adulthood (Golden et al., 1999). NRTN mRNA is also detected in ventral midbrain in adulthood, but in lower level than receptor mRNA (Golden et al., 1999). Moreover, NRTN mRNA but not GFRα2 is detected in postnatal striatum (Widenfalk et al., 1997). This is in line with the theory of NRTN as a target-derived neurotrophic factor. However, the localization of endogenous NRTN protein has not been studied.

As GDNF and other GFLs, also NRTN is produced as a precursor protein. Cleavage of the pro-region might be an important regulatory step in NRTN signaling, since pro-NRTN is not able to initiate the

signaling via GFRα1 nor GFRα2 complexed with Ret (Fjord-Larsen et al., 2005). Moreover, deletion of the pro-region improves the secretion of NRTN (Fjord-Larsen et al., 2005). Even though very little is known about the effects of pro-NRTN, mutations in the pro-region have been associated with Hirschprung's disease, a clinical condition caused by defects in enteric nervous system (Doray et al., 1998).

Mature NRTN is active as a homodimer and binds preferentially to the glycophosphatidylinositol-anchored GFRα2 receptor (Baloh et al., 1997; Klein et al., 1997). In addition to Ret, NRTN/GFRα2-complex can initiate the signaling via neural cell adhesion molecule (Paratcha et al., 2003). NRTN can also signal GFRα and Ret –independently via transmembrane heparan sulfate proteoglycan syndecan-3 (Bespalov et al., 2011). Unlike GDNF, NRTN might be able to signal via integrin β-1 too (Schmutzler et al., 2011). Another difference in the GDNF and NRTN signaling has been detected in the kidney. Whereas GDNF is a paracrine morphogen, NRTN has been shown to exert its functions also in an autocrine manner in developing kidney ducts (Davies et al., 1999).

Unlike GDNF knockout mice, NRTN knockout mice are viable and fertile (Heuckeroth et al., 1999). The mice have defects in the enteric nervous system, but unlike GDNF knockout mice, NRTN knockouts have morphologically and functionally normal kidneys. Furthermore, NRTN knockout animals have deficits in parasympathetic system and sensory systems, but no abnormalities were characterized in the central nervous system (Heuckeroth et al., 1999). Thus, other neurotrophic factors and signaling pathways might compensate for the lack of NRTN signaling.

Artemin and persephin

Artemin was identified based on its homology with the NRTN protein sequence (Baloh et al., 1998). In an in vitro assay, artemin promoted the survival of neurons from both peripheral nervous system and midbrain dopaminergic neurons as efficiently as GDNF or NRTN (Baloh et al., 1998). These observations have led to test the efficacy of artemin in the 6-OHDA model of PD, where lentiviral vector-mediated overexpression of artemin provided neuroprotective effects against 6-OHDA-induced neurotoxicity (Rosenblad et al., 2000). Persephin was identified using polymerase chain reaction with primers recognizing homologous sequences in GDNF and NRTN genes (Milbrandt et al., 1998). In contrast to artemin and other GFLs, persephin does not promote survival of peripheral neurons in vitro (Baloh et al., 1998; Milbrandt et al., 1998). Nonetheless, persephin promotes survival of midbrain dopaminergic neurons both in vitro and in vivo (Milbrandt et al., 1998). Recently, lentivirus-mediated overexpression of persephin was shown to be neuroprotective in the rat 6-OHDA model of PD (Yin et al., 2015).

Therapeutic potential of GDNF and NRTN: Protein infusion studies

Neurotrophic factors do not pass blood-brain barrier and they need to be delivered intracranially. Single intranigral injection of GDNF and NRTN in 6-OHDA animal model of PD showed great potential in protecting and restoring the dopamine environment of the nigrostriatal pathway. Both neurotrophic factors promoted the survival of dopaminergic neurons as well as striatal dopamine (Hoffer et al., 1994; Horger et al., 1998). Moreover, repeated intranigral administration of both neurotrophic factors was well tolerated and provided almost complete protection of TH-immunoreactive cells in animals (Horger et al., 1998). However, intranigral injection of GDNF did not spare striatal innervation, and the effects were limited to nigral cell bodies (Sauer et al., 1995). Similar effects were observed in mouse MPTP model of PD (Tomac et al., 1995a). In contrast, injection of GDNF to striatum was able to protect the nigral cell bodies and striatal dopaminergic neurites as well as provide functional benefits in both 6-OHDA rat and MPTP mouse models of PD (Tomac et al., 1995a; Kirik et al., 2000a). These results together with the observation that striatally administered GDNF protein is retrogradely transported to the SNpc (Tomac et al., 1995b; Ai et al., 2003) supported the change of the administration paradigm

to intrastriatal delivery. Indeed, repeated striatal administration of GDNF was more efficient in restoring the striatal TH-immunoreactive fiber density than nigral administration (Tomac et al., 1995a; Kirik et al., 2000a). Interestingly, repeated intranigral infusion of NRTN was proven to be as potent as GDNF in protecting neurons in SN (Horger et al., 1998), but when delivered intrastriatally, NRTN was less effective in protecting nigral neurons than GDNF and with intraventricular delivery, NRTN was completely ineffective (Rosenblad et al., 1999). This might be due to the diffusion differences since NRTN binds tightly to heparin sulfates of the extracellular matrix in the brain tissue, as well as poor solubility at neutral pH (Rosenblad et al., 1999; Hamilton et al., 2001; Bespalov et al., 2011).

Continuous infusion of GDNF to putamen or ventricle was also beneficial in the non-human primate MPTP model of PD (Grondin et al., 2002). GDNF increased putaminal dopamine concentration and the density of striatal TH-immunoreactive fibers as well as the number of nigral dopaminergic neurons (Grondin et al., 2002; Ai et al., 2003). The positive results of GDNF protein infusions led to clinical trials (table 2). Intraventricular infusion of GDNF did not improve the clinical status of the patients and adverse side effects, such as weight loss and nausea were reported (Nutt et al., 2003). In contrast, intraputaminal delivery of GDNF protein improved the clinical status of the patients without major adverse effects (Gill et al., 2003). However, the results did not replicate in the following study, where the patients also developed antibodies against GDNF (Lang et al., 2006) and further trials were halted.

Table 2. GDNF clinical trials.

Original study	Administration	Results	Follow-up
(Nutt et al., 2003) Phase I/II	Unilateral ventricular monthly bolus	No clinical improvement Adverse effects	No evidence of nigrostriatal regeneration or GDNF diffusion (Kordower et al., 1999)
(Gill et al., 2003) Phase I	Bilateral (1 patient unilateral) putaminal continuous infusion	Clinical improvement No adverse effects	Clinical improvement, F-DOPA uptake increased, medication-induced dyskinesia decreased (Patel et al., 2005) Increase in TH+ innervation putamen (Love et al., 2005)
(Lang et al., 2006) Phase II	Bilateral putaminal continuous infusion	No clinical improvement Antibodies detected	GDNF concentrated around the catheter (Salvatore et al., 2006) Statistically underpowered study (Hutchinson et al., 2007)
(Slevin et al., 2005) Phase I	Unilateral putaminal continuous infusion	Clinical improvement No adverse effects	Halted when r-metHuGDNF withdrawn from market All improvements lost in 1 year (Slevin et al., 2007)
Medgenesis[a] Phase II	Bilateral putaminal intermittent infusion	No improvement	NA
NCT01621581[b] ongoing Phase I/II	AAV-GDNF putamen	NA	NA

[a]Press release 7.6.2016 (http://www.medgenesis.com/news.htm)
[b]Clinicaltrials.gov identifier

Besides intracranial infusion, intranasal administration is another route to deliver therapeutic proteins to the brain. The advantages of intranasal delivery are that it is non-invasive, bilateral, bypasses blood-brain barrier, and the minimal absorption into systemic circulation reduces the risk of peripheral side effects. On the other hand, only a small fraction of intranasally administered protein reaches the brain and therefore the therapeutic molecules have to be administered in high dose and in small volume. Thus, the solubility of the molecule is essential. Intranasal delivered GDNF enters the brain via olfactory and trigeminal pathways and increases GDNF levels in the whole brain, including striatum and SN one hour after the delivery in rats (Bender et al., 2015). Indeed, intranasal administration of 50 µg of GDNF one hour prior to 6-OHDA protected dopaminergic cell bodies in SN but did not have an effect on

striatal innervation or behavior (Migliore et al., 2014). However, the translation of rodent data to humans is difficult due to anatomical differences (van Woensel et al., 2013).

Viral vector delivery of neurotrophic factors

Gene therapy provides long-term expression of the therapeutic protein in the target tissue, and therefore it has been widely studied in preclinical models of PD. Both viral vectors and DNA nanoparticles have been tested with positive effects (Kirik et al., 2000b; Fletcher et al., 2011). With viral vector delivery, the effects of neurotrophic factors were site-dependent (Kirik et al., 2000b). Striatal overexpression of GDNF was observed to promote functional recovery of 6-OHDA-treated animals, whereas nigral overexpression was only protecting nigral cell bodies (Kirik et al., 2000b). Similar results were obtained with CERE-120, adeno-associated virus serotype 2 (AAV2)-based vector encoding NRTN (Gasmi et al., 2007). Striatal delivery of CERE-120 protected both striatal dopaminergic innervation as well as nigral cell bodies from 6-OHDA-induced cytotoxicity. It is evident, that GDNF and NRTN can both protect dopaminergic neurons from cytotoxic insults. However, overexpression of GDNF did not attenuate viral vector-mediated α-synuclein-induced neurodegeneration in α-synuclein overexpressing rats (Decressac et al., 2011). The lack of neuroprotective effects might be due to α-synuclein-induced downregulation of transcription factor Nurr1 and its downstream target Ret, disrupting the GDNF signaling (Decressac et al., 2012). Nevertheless, the translational value of these findings can be questioned, since simultaneous overexpression of two proteins might cause ER stress in vulnerable nigral neurons leading to UPR, masking the neuroprotective effects (Hoffer and Harvey, 2011).

Since promising results of viral delivery in rodent models were replicated in non-human primate models of PD (Kordower et al., 2000; Palfi et al., 2002; Kordower et al., 2006a; Kells et al., 2010), clinical trials were started with CERE-120 (table 3). The first results from the trials were positive: one year after the intraputaminal viral vector delivery patients did show improvement in their condition. In addition, no adverse side effects were reported (Marks et al., 2008). To improve the bioavailability of NRTN, in following clinical trials CERE-120 was delivered both intraputaminally and nigrally. Although the results were declared negative in the trial, re-analysis of the data confirmed a small subpopulation of the patients to have benefitted from the therapy. The therapeutic efficacy of NRTN was highest in patients which were diagnosed with PD less than five years ago (Bartus, 2015; Olanow et al., 2015). Thus, this is in line with analysis of the PD brain showing clear reduction in striatal dopaminergic fibers already five years after diagnosis (Kordower et al., 2013) and following impairment in axonal transport (Bartus et al., 2011). A post-mortem sample four years after CERE-120 delivery revealed the patient to have some NRTN-immunoreactivity in both SN and putamen (Bartus et al., 2015). Currently, safety and efficacy of CERE-120 are being evaluated in patients with moderately advanced PD (clinicaltrials.gov identifier NCT00985517), as well as the safety of AAV2-GDNF is being tested in more advanced PD patients (clinicaltrials.gov identifier NCT01621581).

Table 3. Neurturin clinical trials.

Original study	Administration	Results	Follow-up
(Marks et al., 2008) Phase I	Bilateral putaminal	Clinical improvement No adverse effects	Lack of anterograde transport (Bartus et al., 2015)
(Marks et al., 2010) Phase II	Bilateral putaminal	No clinical improvement Adverse effects	Lack of anterograde transport (Bartus et al., 2011; Bartus et al., 2015) Subset of patients improvement after 15-18 month follow-up (Olanow et al., 2015)
(Bartus et al., 2013) Phase I/II	Bilateral putaminal + nigral	Clinical improvement No adverse effects	NA
(Olanow et al., 2015) Phase II	Bilateral putaminal + nigral	No clinical improvement No adverse effects	Clinical improvement in a subset (<5 years of diagnosis) of patients (Bartus, 2015)
NCT00985517[a] ongoing Phase I/II	Bilateral putaminal + nigral	NA	NA

[a]clinicaltrials.gov identifier

Ex vivo gene therapy of neurotrophic factors has also been applied in preclinical studies. Bone marrow-derived mesenchymal stem cells provide an attractive approach to enhance GDNF signaling in the brain since the cells can be isolated from an autologous source, the patient himself, genetically manipulated to overexpress therapeutic proteins, and implanted in the brain. Striatal implantation of mesenchymal stem cells genetically manipulated to express GDNF have been shown to protect striatal innervation from a 6-OHDA-induced cytotoxic insult and to improve the rotational asymmetry induced by 6-OHDA administration (Glavaski-Joksimovic et al., 2010). In another study, neural stem cells expressing NRTN were engrafted to striatum (Liu et al., 2007). NRTN significantly reduced the rotational imbalance in the animals after 6-OHDA administration several months after the cytotoxic insult as well as increased striatal levels of dopamine and its metabolites. Moreover, retrograde transportation of NRTN to SN protected the cell bodies from cell death (Liu et al., 2007).

Endogenous GDNF and dopaminergic system

Increasing the expression of endogenous GDNF also has neuroprotective effects against toxin-induced neurodegeneration. Laganiere and colleagues used AAV-mediated delivery of artificial GDNF transcription activator to enhance the striatal expression of GDNF. Although activation of GDNF gene provided significant functional protection against 6-OHDA-induced motor impairments, the protective effect was less clear in SNpc neurons (Laganiere et al., 2010). Another approach to increasing the expression of endogenous GDNF is to inhibit the transcription of the 3' untranslated region of the Gdnf gene (Kumar et al., 2015). These GDNF hypermorph mice overexpressing GDNF from the native locus have increased striatal dopamine level and increased number of nigral dopaminergic neurons and striatal nerve terminals. Although overexpression of GDNF did not protect nigral dopaminergic neurons from the lactacystin-induced degeneration, the striatal dopamine level was preserved in GDNF hypermorph mice. On the other hand, the GDNF hypermorph mice are more susceptible to 6-OHDA cytotoxicity than WT mice due to increased DAT activity (Kumar et al., 2015).

Effects of exogenous GDNF on mitochondrial dysfunction

Most of the data regarding the therapeutic potential of GFLs has been gathered in 6-OHDA or MPTP animal models of PD and the focus has been on the dopaminotrophic effects, survival of the dopaminergic neurons and neurites. Hence, less is known about the effects of GFL signaling on other aspects of PD, such as mitochondrial defects. Several studies are pointing at a link between GFL signaling and mitochondrial function. Striatal administration of GDNF to MPTP-treated mice regulates several PD-associated genes, such as DJ-1 and UCHL-1 (Hong et al., 2009). In the 6-OHDA animal model, administration of GDNF prior to toxin reduces oxidative stress markers in the striatum, suggesting it reduced the formation of ROS (Smith and Cass, 2007). In vitro experiments with neuroblastoma cells have shown the absence of DJ-1 to suppress the expression of Ret (Foti et al., 2010). When the neuroblastoma cells lacking parkin were treated with GDNF and GFRα1, the reduced cellular ATP levels were restored (Meka et al., 2015). Similar effects were observed with cells lacking PINK1, where stimulation of endogenous Ret with GDNF and soluble GFRα1 recovered mitochondrial fragmentation and further, mitochondrial dysfunction (Klein et al., 2014). However, in MitoPark mice, which exhibit respiratory chain impairment in midbrain dopaminergic neurons leading to a progressive degeneration of nigrostriatal pathway (Ekstrand et al., 2007), application of exogenous GDNF did improve the behavioral deficits but did not have an effect on the neuronal degeneration. Thus, in this model, GDNF was not able to fully rescue the mitochondrial dysfunction-induced phenotype of the animals (Sterky et al., 2013).

Hence, the positive effects of exogenous GDNF and NRTN in the animal models of PD have not translated to human trials. Several reasons have been suggested for this, such as sub-optimal design of the catheter (table 2, Salvatore et al., 2006) and inadequate posttranslational modifications of the proteins (Hoane et al., 2000; Fjord-Larsen et al., 2005; Piccinini et al., 2013). The difference in the brain volume between humans and non-human primates as well as differences in administration paradigm and assessment of the severity of parkinsonism make the translation more difficult (Kordower et al., 1999; Nutt et al., 2003). This raises the question of the predictive validity of current animal models of PD. As discussed in section 2.2., the animal models fail to simulate the complexity of pathological events associated with PD, and might therefore give “false positive” results. Conversely, failure to exhibit neuroprotective effects in one animal model should be interpreted cautiously. The neuroprotective and neurorestorative effects of GFLs have been studied mainly in MPTP and 6-OHDA models of PD, and only few studies have been conducted in other models. However, different models give insight to different aspects of PD and might therefore give valuable information for the drug development and for the basic biology of the neurotrophic factors.

3 AIMS OF THE STUDY

Due to their neuroprotective and neurorestorative properties, neurotrophic factors have been considered as a promising new therapy for Parkinson's disease. The purpose of this thesis was to investigate the neuroprotective and neurorestorative properties of novel and less studied isoforms of neurotrophic factors NRTN and GDNF.

The specific aims of the study were as follows:

- To study the neuroprotective properties of novel neurturin variants with decreased heparin binding properties. The poor solubility and high affinity of NRTN to heparan sulfated proteoglycans of the extracellular matrix have been suggested to hamper the diffusion of NRTN in the brain and further, the therapeutic effects. Thus, we investigated whether improved diffusion capacity enhances the therapeutic effects of the protein.

- To develop a stable partial 6-OHDA rat model of Parkinson's disease with high success rate. Since animals recover spontaneously from 6-OHDA cytotoxic insults, improving the stability of the animal model improves the reliability of the obtained results and importantly, decreases the number of experimental animals used in the studies.

- To compare the effects of conserved GDNF splice isoforms in the non-lesioned striatum and in partial 6-OHDA lesion model of Parkinson's disease. Full-length α-GDNF and corresponding mature GDNF are secreted constitutively whereas shorter β-GDNF and corresponding mature GDNF are secreted activity-dependently. Hence, the distinctive effects of the isoforms on the nigrostriatal dopaminergic circuitry were evaluated.

4 MATERIALS AND METHODS

4.1 Animals

Male Wistar rats (Harlan/Envigo, The Netherlands) were used in all studies. Rats were housed in groups of 3-5 in 12h/12h light/dark cycle, with standard rodent chow and water ad libitum. Experimental design and procedures regarding animals were reviewed and accepted by the National Experiment Board (ESAVI/5459/04.10.03/2011 and ESAVI/7812/04.10.07/2015) and conducted according to EU regulation (EU directive 2010/63/EU) and Finnish legislation (Finnish act on the protection of animals used for scientific or educational purposes [497/2013] and the Government decree on the protection of animals used for scientific or educational purposes [564/2013]).

4.2 Stereotaxic surgeries

All stereotaxic procedures were done under isoflurane anesthesia (induction 4-4.5%, sustenance 2.5% isoflurane). Tramadol (1mg/kg, s.c. study I and II) or carprofen (5 mg/kg, s.c., study III) were used as a post-operative analgesic. 6-OHDA injection coordinates (according to Paxinos and Watson 2005) and microinjection syringes used in experiments are designated in table 4. Same coordinates were used to infuse neurotrophic factors (study I, 5 µg of neurturin variants or GDNF in 10 µl) or viral vectors (study III, AAV, 3 x 1.5 µl, Figure 7).

Table 4. 6-OHDA injection paradigms used in the experiments.

Dose	Volume	Coordinates			Needle	Study
		A/P	L/M	D/V		
3 x 1 µg	3 x 1.5 µl	+1.6	-2.8	-6.0	WPI 33G	II
		0.0	-4.1	-5.5		
		-1.2	-4.5	-5.5		
3 x 2 µg	3 x 1.5 µl	+1.6	-2.8	-6.0	WPI 33G	II, III
		0.0	-4.1	-5.5		
		-1.2	-4.5	-5.5		
3 x 3 µg	3 x 1.5 µl	+1.6	-2.8	-6.0	WPI 33G	II
		0.0	-4.1	-5.5		
		-1.2	-4.5	-5.5		
2 x 10 µg^{prog}	2 x 2.5 µl	+1.6	+2.2	-5.5	Hamilton 26G	II
		-0.4	+3.0	-5.5		
2 x 10 µg^{reg}	2 x 2.5 µl	+1.6	+2.2	-5.5	Hamilton 30G	II
		-0.4	+4.0	-5.5		
1 x 20 µg	5 µl	+1.0	+2.7	-5.5	Hamilton 26G	II
3 x 7 µg	3 x 1.75 µl	+1.6	+2.2	-5.5	WPI 33G	II
		0.0	+3.5	-5.5		
		-1.2	+3.9	-5.5		
1 x 28 µg	7 µl (4 x 1.75 µl)	+1.0	+2.8	-6.0	Hamilton 26G	I
				-5.5		
				-5.0		
				-4.4		

2 x 10 µg^{prog}= Injection with 26G Hamilton syringe, progressive rotational behavior (see results section 5.2)
2 x 10 µg^{reg}= Injection with 30G Hamilton syringe, regressive rotational behavior (see results section 5.2)
WPI, World Precision Instruments

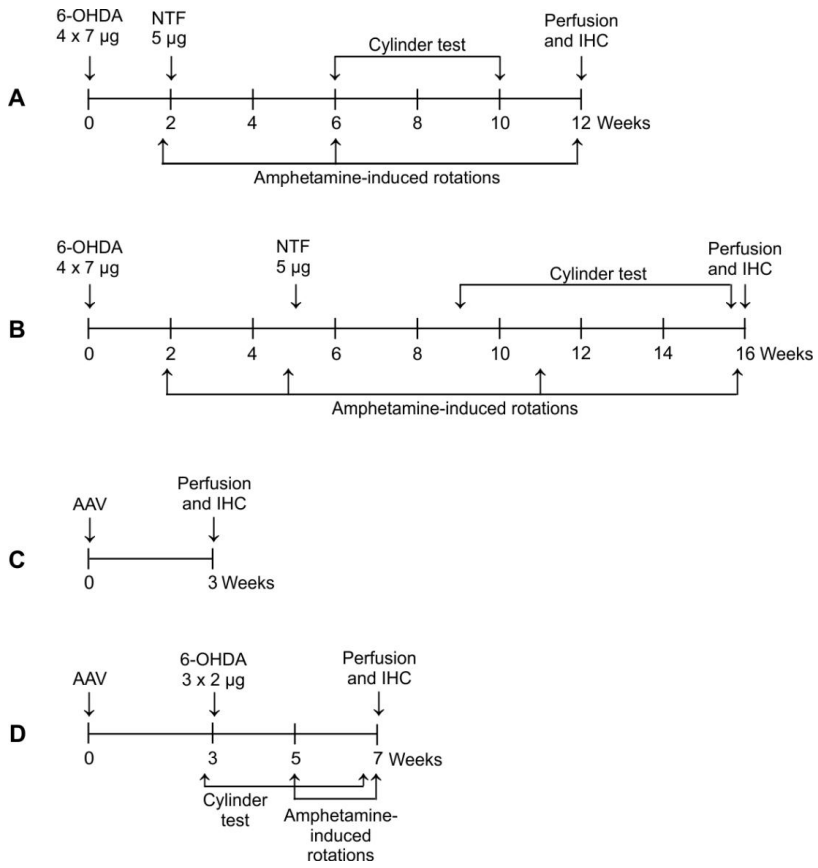

Figure 7. Experimental design. Neurotrophic factors were delivered intracranially as a protein infusion either A) two weeks (study I) or B) five weeks (unpubl. results) after 6-OHDA administration. With AAV1 vector delivery, the transgene expression was allowed to continue for three weeks before evaluating the effects in C) non-lesioned animals or D) inducing neurodegeneration with 6-OHDA (study III). 6-OHDA, 6-hydroxydopamine; AAV, adeno-associated virus; IHC, immunohistochemistry; NTF, neurotrophic factors.

4.3 Behavioral assays

4.3.1 Amphetamine-induced rotations

Rotational asymmetry was measured after unilateral 6-OHDA injection. 2.5 mg/kg (s.c.) of amphetamine sulfate was administered to induce ipsilateral rotational behavior (Ungerstedt and Arbuthnott, 1970) after a 30 minute habituation period. Full, uninterrupted 360° turns were counted for 120 minutes in automatic rotometer bowls (MedAssociates, Inc. St. Albans, VT).

4.3.2 Cylinder test

Cylinder test was used to assess asymmetry in spontaneous forelimb use. Freely moving rats were filmed for five minutes in a plexiglass cylinder (diameter 20 cm) and the contacts made by each forepaw with the cylinder wall were scored by blinded observer. Only weight-bearing touches were considered as touches. In study I, the cylinder test was conducted in normal light, but in study III, the

test was carried out under red light to increase the activity of the animals. The animals were let to adjust to red light for 30 minutes before testing.

4.4 Immunohistochemistry

For immunohistochemistry, the animals were anesthetized with pentobarbital and perfused transcardially with PBS and 4% paraformaldehyde. The removed brains were cut into 40 µm thick sections in series of six and stored at -20°C.

The immunohistochemistry was performed on free-floating brain sections. After washing, the endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide, and in DAT-staining, additional incubation at 80°C was conducted to enhance the antigen recognition. The sections were incubated with 4% BSA and 0.1% Triton X-100 in PBS to reduce non-specific binding before incubation with desired primary antibody overnight (table 5). Following the incubation with suitable biotinylated secondary antibody, the staining was reinforced with avidin-biotin complex and visualized with 3', 3'-diaminobenzidine.

In fluorescence staining, the sections were incubated with blocking buffer after washing, incubated with primary antibody overnight, and mounted on microscope slides after incubation with secondary antibody conjugated with a fluorescent dye.

Table 5. Antibodies and their dilutions used in the experiments.

Antigen	Host	Producer	Catalogue number	Dilution
Fos	Rabbit	Santa Cruz	sc-52	1:1000
Dopamine transporter (DAT)	Rat	Millipore	MAB369	1:2000
Glial cell line-derived neurotrophic factor (GDNF)	Goat	R & D Systems	AF-212-NA	1:3000
Green fluorescent protein (GFP)	Rabbit	Life Technologies	A11122	1:2000
Tyrosine hydroxylase (TH)	Mouse	Millipore	MAB318	1:2000

4.5 Morphometric analysis

4.5.1 Optical density

The optical density of TH-immunoreactive fibers was analyzed from three (study II), six (study III), or nine (study I) adjacent sections. The density was determined with ImagePro (studies I and III, Media Cybernetics, Rockville, MD, USA), or ImageJ (study II, NIH, Bethesda, MD, USA) from images acquired either with a digital camera (Nikon Corp., Tokyo, Japan) attached to a microscope (study I) or with a digital microscope slide scanner (studies II and III, 3D Histech, Budapest, Hungary). Corpus callosum was used to correct the values for background staining, as it is devoid of staining signal. Data are expressed as a percentage from the intact side.

4.5.2 Cell counting

The number of TH-immunoreactive cells in the SNpc was estimated from six adjacent sections with Matlab (studies II and III) or from nine adjacent sections with StereoInvestigator (study I, MicroBrightfield, Williston, VT, USA) by blinded observer. In the Matlab method, the cells were recognized from the digitalized images obtained with 3D Histech scanner (3D Histech, Budapest, Hungary) based on their size in pixels and signal intensity. The cell size was user-defined based on the size of single cells in example images and kept constant throughout the whole analysis. This way small

objects were excluded from the cell counts and the area covered by larger objects consisting of overlying cells was divided by the size of the single cell, thus estimating how many cells were clumped together. Since the signal intensity varies between the samples, the intensity threshold was defined individually for every sample based on the signal intensity of single cells. The background devoid of cells and staining signal was kept at zero. StereoInvestigator, on the other hand, is based on stereology, and the cell counting was done with optical fractionator combined with dissector principle and unbiased counting rules from the brain sections mounted on microscope slides (West et al., 1991). In both cases, the results are given as a percentage of the unlesioned side.

4.6 Other methods

Other methods used in the studies are presented in table 6. More detailed description of the methods can be found in the original publications and their supplements. Methods the author has been involved in are marked with a *.

Table 6. Other methods used in the study.

Method	Original study
Cell-based binding assay	I
Cell culture	I
Cloning	I, III
Computational modelling	I
Diffusion assays *in vivo**	I
ELISA*	III
GFRα affinity chromatography	I
Heparin affinity chromatography	I
Immunocytochemistry	I
Kidney *ex vivo* organogenesis	I
N-terminal sequencing	I
PCR mutagenesis	I
Preparation of viral vectors	III
Programming	II
Protein diffusion assay in non-human primates	I
Protein production	I
Proteolytic resistance	I
Purification of protein	I
Purification of viral vectors	III
RET phosphorylation assay	I
RT-PCR	I
Survival assay on embryonic dopaminergic neurons *in vitro*	I
Transient cell transfection	I, III
Western blotting	I

4.7 Statistical analysis

Data are expressed as a mean ± standard error of the mean (SEM). Statistical analyses were done by SPSS (IBM). Differences between the treatment groups are analyzed with one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD), Tukey's, or Dunn's multiple comparison *post hoc,* or two-way ANOVA or repeated measures ANOVA, or paired t-test. In non-parametric situations, Kruskal-Wallis test was conducted with Bonferroni *post hoc* analysis. Correlation analysis was conducted using Pearson correlation test. Results were considered significant at level $p \leq 0.05$.

5 RESULTS

5.1 Cell counting method

A new method for counting the TH-positive cells in the SNpc was set up and validated. Previously used stereology-based method combined with optical dissector and fractionator enabled the cells to be recognized irrespective of their shape, size or orientation. In contrast, the new Matlab algorithm-based method recognizes the cells by their size in pixels in digitalized images. Despite this methodological difference, the results obtained with the methods have a high correlation ($R=0.925$, $F_{1,6}=35.565$, $P<0.001$).

5.2 Stable 6-OHDA model of Parkinson's disease

5.2.1 Rotational differences between 6-OHDA doses

6-OHDA is a classical animal model of PD. By using a stable animal model with high success rate, the number of animals needed to obtain reliable data is reduced. In study I we used 1 x 28 µg dose, where four 7 µg depots of 6-OHDA were injected to the same site, above each other (table 4). When the animals were rotated two weeks later, only 54% of them rotated sufficiently (table 7) (in study I the limit was minimum of 220 turns in 120 mins) and were selected for the neuroprotection study (hence the name 1 x 28 μg^{2week}). The animals were assigned randomly to the treatment groups, and the groups were balanced based on the rotations so that all groups included animals with high rotations and animals with low rotations. Thus, the average of the rotations was at similar level in all treatment groups. Animals, which did not rotate two weeks after 6-OHDA injection, were re-assessed three weeks later on week five to see whether they developed a lesion later (hence the name 1 x 28 μg^{5week} group). This time only 49% of the remaining animals rotated in the assay (table 7). These animals were divided into treatment groups in similar manner as previously based on their rotational behavior (neurorestoration study).

Considering that in study I only about half of the animals injected with 6-OHDA developed a lesion during the first two weeks and were included in the original neuroprotection experiment, the used injection paradigm was not optimal. In study II, we compared seven different 6-OHDA injection paradigms (table 4) used in our previously unpublished experiments to find out what are the crucial elements to create a stable animal model with high success rate. The data was re-analyzed by the amphetamine-induced rotations and immunohistochemistry. The control group data from study I neuroprotection experiment (1 x 28 μg^{2week}) and neurorestoration experiment (1 x 28 μg^{5week}) were also included to the comparison in this thesis. Since the data is a compilation of control groups from different experiments, the time points vary between the groups. However, all groups were rotated during weeks 1-2 to see whether the lesion was successful or not. Animals rotating more than 100 turns in two hours were considered to have a lesion (study II; Bäck et al., 2013b), exception in study I, where the limit was 220 turns in 120 minutes). The percentage of animals with a successful lesion was considered as a measure for replicability (success rate) of the model (table 7). In an optimal model, the success rate would be high and most 6-OHDA-injected animals would develop lesion and thus be included in the experiments.

Table 7. Immunohistochemistry results of different lesion paradigms.

Treatment group	Category	TH-ir str (% of the intact side)	DAT-ir str (%of the intact side)	TH-ir SNpc (% of the intact side)[a]	Success rate (%)	
					Start	End
3 x 3 µg	Progressive	21 ± 5	22 ± 3	29 ± 5	88	100
2 x 10 μg^{prog}		27 ± 5	21 ± 4	23 ± 4	91	100
3 x 7 µg		30 ± 2	17 ± 2	22 ± 3	100	100
3 x 1 µg	Stable	31 ± 9	24 ± 6	43 ± 6	63	80
3 x 2 µg		28 ± 6	27 ± 5	36 ± 7	75	75
1 x 28 μg^{2week}		13 ± 4	20 ± 4	31 ± 6	54	80
1 x 28 μg^{5week}		NA	NA	NA	49	57
2 x 10 μg^{reg}	Regressive	66 ± 7	58 ± 6	71 ± 0,2	100	43
1 x 20 µg		51 ± 6	43 ±5	61 ± 6	100	70

2 x 10 μg^{prog}= progressive rotations, Hamilton 26 G

2 x 10 μg^{reg}= regressive rotations, Hamilton 30 G

1 x 28 (4 x 7) μg^{2week}= 2 week group (lesion limit 220 turns in 120 mins)

1 x 28 (4 x 7) μg^{5week} = 5 week group (lesion limit 220 turns in 120 mins)

Success rate: number of animals rotating >100 turns in 120 mins

[a]TH-ir cells analyzed with Matlab

DAT-ir, dopamine transporter-immunoreactivity; SNpc, substantia nigra pars compacta; str, striatum; TH-ir, tyrosine hydroxylase-immunoreactivity.

Ideally, unbiased assessment, such as d-amphetamine-induced rotations, should correlate with dopamine depletion. Different paradigms were categorized as progressive, stable or regressive based on their rotational behavior (figure 8). Although the rotational behavior has been measured at different time points, the difference between the categories is clear. In groups categorized as progressive, the rotations were increased 100-400% (3 x 3 µg 112%, 2 x 10 μg^{prog} 154%, and 3 x 7 µg 387%) from the first time point rotations (week 1-2). These groups had also high success rates in both first and last measured time point (table 7). In stable rotation groups the changes were more subtle (3 x 1 µg -17% and 3 x 2 µg 24% , 1 x 28 μg^{2week} 31%, and 1 x 28 μg^{5week} 26%). In 3 x 1 µg and 3 x 2 µg groups the success rate was at moderate level both in first and last time point (table 7). On the other hand, 1 x 28 μg^{2week} and 1 x 28 μg^{5week} groups had low success rates but stable rotational behavior. In regressive groups the rotational behavior decreased from the first time point (-84% 2 x 10 μg^{reg} and -51% 1 x 20 µg group). These groups however showed high success rates at the first time point, but the in the last time point (2 x 10 μg^{reg} week 10 and 1 x 20 µg week 8) only 43% and 70% of the animals rotated over the limit of 100 turns in two hours.

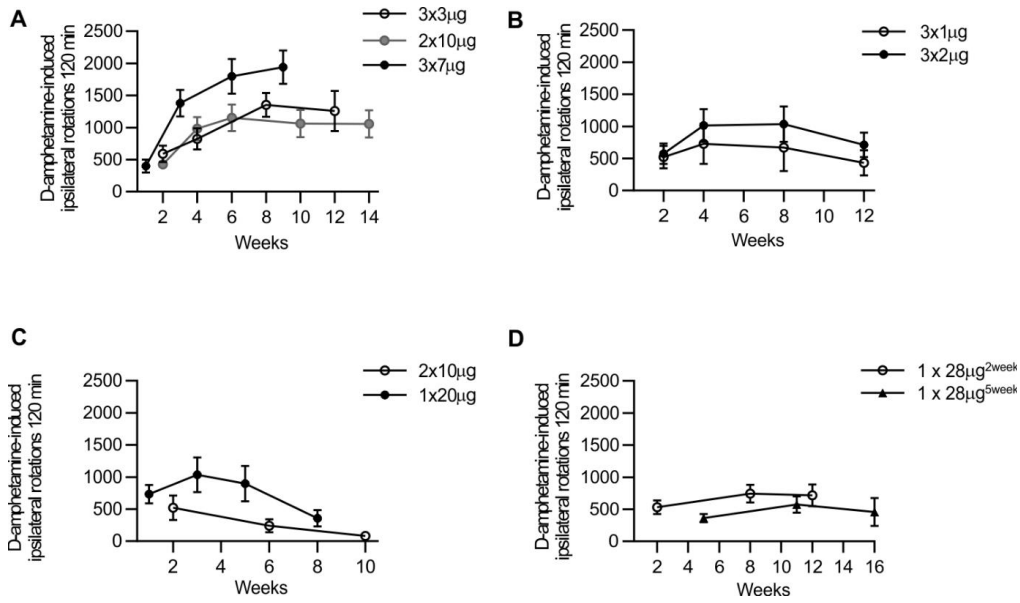

Figure 8. In study II, the lesion groups were categorized as A) progressive, B) stable, or C) regressive based on their rotational behavior. D) The lesion paradigm used in study I also produced stable rotational behavior.

Interestingly, we observed a dose-response in the low-dose 6-OHDA treated groups in the last measured time point, week 12 (3 x 1 µg 432 ± 198 turns, 3 x 2 µg 712 ± 189 turns, and 3 x 3 µg 1258 ± 315 turns in 120 mins), which is in line with previous studies showing 6-OHDA induced lesions to be dose-dependent (Przedborski et al., 1995; Lee et al., 1996; Bäck et al., 2013b). This was however not observed with higher doses. Since 3 x 2 µg injection paradigm produced a stable rotational behavior with good success rate, it was chosen for the following study III.

5.2.2 Immunohistochemistry

Surprisingly, all 6-OHDA administration paradigms induced the same magnitude of lesion of the nigrostriatal pathway (table 7), with the exception of 1 x 20 µg and 2 x 10 μg^{reg} groups, in which the lesion was much smaller than in other groups. The density of DAT-immunoreactive fibers was also at a similar level in all progressive and stable lesion groups and higher in the regressive lesion groups. Also, the TH-immunoreactivity in the striatum reflected the number of TH-immunoreactive cells in SNpc since there was a high correlation between the measures ($R=0.785$, $p<0.0001$). The correlation between DAT-immunoreactive fibers and TH-immunoreactive cells in SNpc was even higher (study II, $R=0.838$, $p<0.0001$). Even though 6-OHDA induced loss of striatal and nigral TH-immunoreactivity has been shown to be dose-dependent (Lee et al., 1996; Bäck et al., 2013b), this was not observed in our study (II). Instead, when the toxin was injected into multiple sites (e.g. 3 x 1 µg), the lesion size was comparable to higher doses (e.g. 2 x 10 µg, table 7).

Although the rotational assay has been widely used, the results might be easily over-interpreted (Marin et al., 2006; Meredith and Kang, 2006). As is shown in study II, the rotational behavior does not always reflect the actual loss of TH-immunoreactive cells in SNpc or TH-immunoreactive fibers in the striatum. Despite the difference in the time points when the assay was conducted, there was a clear difference in the rotational behavior between the progressive and the stable groups, but the striatal TH- and DAT-

immunoreactivity was at the same level in all groups (table 7, correlation between rotations and striatal TH-immunoreactivity R=0.536, p<0.0001, correlation between rotations and DAT-immunoreactivity R=0.591, p<0.0001). The correlation between the rotations and the number of TH-immunoreactive cells in SNpc was a little bit higher (R=0.662, p<0.0001).

5.3 Development of NRTN variants with reduced heparin affinity

NRTN and GDNF both bind strongly to extracellular matrix and cell surface heparan sulfated proteoglycans (Hamilton et al., 2001; Bespalov et al., 2011), which can attenuate their diffusion in the brain parenchyma. This is problematic from the therapeutic perspective since neurotrophic factors do not pass the blood-brain barrier and have to be delivered intracranially. Poor diffusion and the following low bioavailability have been suggested to be one of the reasons why the clinical trials with GDNF have produced only mild effects (Salvatore et al., 2006).

5.3.1 Modelling NRTN variants

New NRTN variants were created to improve the solubility, diffusion capacity and bioavailability of the protein in the brain tissue. First, the three-dimensional structure of NRTN, a close homolog of GDNF, was modeled using the structure of GDNF (Eigenbrot and Gerber, 1997; Parkash et al., 2008). In the modeled structure, putative heparin binding sequence BBXB or BBXXB, where B is basic amino acid and X is any amino acid (Cardin and Weintraub, 1989), was localized in the heel region ($_{51}$RRLRQRRRLRRER$_{63}$), where the required positively charged amino acids are aligned pointing to the same direction. Since the heel region included several putative consensus sequences, several mutations were introduced to the region thereby generating several novel variant proteins. Variants N1 and N2 have substitutions of arginine with alanine and N3 is a combination of these mutations, with the exception of the last restoring the conserved last arginine of the sequence. In N4, the heel region was substituted with the heel region of PSPN (ARLQGQG), which does not bind to heparin (Bespalov et al., 2011), and elongated (ALVGS) to match the length of the original NRTN heel sequence.

5.3.2 Biological activity of NRTN variants

For purification and initial testing, the variant proteins were expressed in mammalian chinese hamster ovary cells with N-terminal V5-tag (hence the name NV1-NV4) and with an endogenous ER-signal sequence substituted with ER-signal sequence from IgG (Fjord-Larsen et al., 2005). The native pro-sequence was also removed from the proteins to improve secretion (Fjord-Larsen et al., 2005). A preliminary screen with the Ret-phosphorylation assay showed all the new variants to be biologically active in the presence of either GFRα1 or GFRα2, and heparin affinity chromatography showed all variants to have reduced affinity to heparin. Moreover, the concentration of NV2 and NV4 was higher than the concentration of WT NRTN, NV1 or NV3 in the culture media, although the concentrations of NRTN variants were similar inside the cells. This might reflect the improved resistance to proteolytic degradation of NV4.

For the larger scale production of variant proteins, both pro-sequence and the V5-tag were removed from the variant proteins (variants N1-N4, namely). Untagged proteins were purified with a combination of heparin- and GFRα2-affinity chromatography. Heparin affinity chromatography demonstrated the reduced affinity for heparin of the variant proteins (table 8). However, two of the variants, N1 and N3 aggregated and precipitated during the purification and biochemical assays and in higher concentrations (study I, figure 3). As the proteins were needed in higher concentrations for the in vivo assays, the study focused more on the variants N2 and N4. Similar precipitation problems were also detected with WT NRTN. A binding assay with chinese ovary cells expressing GFRα2, RET and

heparan sulfates showed the variants N2 and N4 to have similar affinity to the GFRα2/RET receptor complex as WT NRTN (table 8). Moreover, while the WT NRTN attached to the cell surface of the cells lacking the GFRα-receptors, N4 did not. If GFRα-receptors were present on the cell surface, both WT NRTN and N4 attached to the cell surface. When the cell surface was lacking both heparan sulfates and GFRα-receptors, neither WT NRTN nor N4 attached to the cell surface (study I, figure 6).

Table 8. Properties of neurturin variants.

Protein	Heparin affinity [NaCl] M	Cell binding assay IC_{50} (nM)	Diffusion rat[a] (mm^3)	Diffusion cynomolgus monkey[b] (mm^3)	Diffusion marmoset monkey[c] (mm^3)
WT NRTN (commercial, e.coli)	1.07	NA	57	-	-
WT NRTN (CHO)	1.08	4.65	-	-	-
WT GDNF (commercial, e.coli)	-	-	-	619	18.4
N1	0.97	-	-	-	-
N2	0.56	3.13	126	538	-
N3	0.56	-	-	-	-
N4	0.48	1.42	217	1228	106.1

[a]In rat diffusion assay, 5 µg of protein was injected to the striatum.
[b]In cynomolgus monkey diffusion assay, equimolar amounts of protein (GDNF 225 µg/225 µl, N2 170 µg/170 µl, and N4 170 µg/205 µl) was injected to the putamen.
[c]In marmoset monkey diffusion assay, 5 µg of protein was injected to the putamen.

Ret-phosphorylation assay revealed that the variants are able to activate Ret in a dose-dependent manner similar to WT NRTN (study I, figure 4). Another dose-dependent assay, in vitro survival assay on dopaminergic neurons from mouse midbrain (E13.5), showed similar patterns between the NRTN variants and WT NRTN (study I, figure 5A). Interestingly, the neurons express only GFRα1, and not GFRα2 (Cacalano et al., 1998), thus suggesting the NRTN variants are able to start a signaling cascade via GFRα1/Ret complex. Next, the biological activity of NRTN variants was tested in tissue culture. In contrast to WT NRTN, both variants N2 and N4 were able to induce extraureteric branching in mouse embryonic kidney cultures (study I, figure 5B). One explanation for this might be the higher stability of variant proteins compared to WT NRTN in the culture medium.

5.3.3 NRTN variants have improved diffusion capacity and neuroprotective effects in vivo

The diffusion capacities of NRTN variants were determined in three different species: rat, cynomolgus monkey, and marmoset monkey (table 8). The results show inevitably how N4 has better diffusion capacity than N2, WT NRTN or GDNF in brain tissue. Especially in cynomolgus monkey brain, the difference is striking. The diffusion assay results further support the in vitro studies showing N2 and N4 to have reduced affinity to heparin and heparan sulfated proteoglycans of the extracellular matrix and cell surface.

To study whether this improved diffusion capacity and protein stability would play a role in the therapeutic efficacy, neuroprotective effects of NRTN variants N2 and N4 were tested in partial 6-OHDA rat model of PD. Compared to previous studies (Lindholm et al., 2007; Piltonen et al., 2009; Voutilainen et al., 2009), a relatively low-dose of 5 µg of proteins were infused to the striatum two weeks after inducing the degeneration of nigrostriatal pathway with 6-OHDA (1 x 28 µg, table 4). The neuroprotective effects were tested with drug-free cylinder test and amphetamine-induced rotations (study I, figure 7A). Since NRTN can precipitate in neutral pH (Hadaczek et al., 2010), GDNF was used as a positive control in the study instead of WT NRTN.

N4 was able to improve the animals' motor performance after 6-OHDA lesion. In the rotation assay, N4 treated animals recovered from the rotational bias induced by the 6-OHDA lesion by week 12 (repeated measures ANOVA $F_{3,40}$=3.2, p=0.033, Fisher LSD post hoc analysis vehicle vs N4 p<0.01, GDNF vs N4 p<0.05, N2 vs N4 p<0.05, table 9). In the drug-free cylinder test on week 10 (8 weeks after protein infusion), N4-treated animals displayed an increased use of the contralateral paw compared to vehicle treated animals (Kruskal-Wallis nonparametric ANOVA p=0.00138, Dunn's multiple comparison vehicle vs N4 p<0.05, table 8). Immunohistochemical findings further supported the behavioral read-outs as the density of TH-immunoreactive fibers was increased in N4-treated animals (one-way ANOVA $F_{3,40}$=4.24, p=0.011, Fisher's LSD post hoc analysis vehicle vs N4 p<0.01, GDNF vs N4 p<0.05, N2 vs N4 p<0.01, table 8). In contrast, GDNF did not increase the density of TH-immunoreactive fibers. In SNpc, all treatments, GDNF, N2 and N4 increased the number of TH-immunoreactive cells (one-way ANOVA $F_{3,40}$=2.951, p=0.0441, vehicle vs GDNF p<0.05, vehicle vs N2 p<0.05, vehicle vs N4 p<0.05, table 9). Thus, all four assays, two behavioral and two immunohistochemical, strongly suggest N4 to have beneficial effects in the partial 6-OHDA model of PD.

Table 9. Effects of NRTN variants on behavioral correlates in the neuroprotection study.

Treatment	Cylinder test (Contralateral paw %)		Rotations (120 min)		TH-ir str (% of the intact side)	TH-ir SNpc[a] (% of the intact side)
	Week 6	Week 10	Week 8	Week 12	Week 12	Week 12
Vehicle	4 ± 1	1 ± 1	778 ± 154	736 ± 188	13 ± 4	18 ± 3
GDNF	7 ± 4	13 ± 6	632 ± 145	558 ± 194	21 ± 4	33 ± 5*
N2	2 ± 1	5 ± 3	688 ± 99	483 ± 176	19 ± 3	36 ± 3*
N4	18 ± 7	29 ± 10 *	310 ± 85	143 ± 69 **#	41 ± 8**#	37 ± 6*

[a]TH-ir number of TH-immunoreactive cells in SNpc analyzed with StereoInvestigator
*p<0.05 (vs vehicle), **p<0.01 (vs vehicle), # p<0.05 (vs GDNF and N2),
SNpc, substantia nigra pars compacta; Str, striatum; TH-ir, tyrosine hydroxylase-immunoreactivity.

5.3.4 Neurorestorative effects of NRTN variants (unpubl. results)

As mentioned in section 5.2.1, the 6-OHDA injection paradigm used in study II (1 x 28 µg) resulted in a low number of lesioned animals. Animals which did not fulfill the rotation criterion (220< turns in 120 min) two weeks after 6-OHDA injection were tested again three weeks later (week five). Rats that had developed a lesion by five weeks after 6-OHDA, were injected with vehicle, GDNF or NRTN variants N2 or N4. The motor performance was tested with cylinder test on weeks 9 and 16 and with amphetamine-induced rotations on weeks 11 and 16 post-lesion (figure 7B). Neither N2 nor N4 showed neurorestorative effects in cylinder test. In the rotation assay, N4 treated animals rotated less than vehicle treated animals on week 11, but the difference did not reach statistical difference (table 10).

Table 10. Behavioral effects of the NRTN variants in the neurorestoration study.

Treatment	Cylinder test (Contralateral paw %)		Rotations (120 min)	
	Week 9	Week 16	Week 11	Week 16
Vehicle	9 ± 5	7 ± 5	573 ± 131	457 ± 220
GDNF	3 ± 2	0 ± 0	452 ± 101	505 ± 185
N2	3 ± 2	14 ± 14	468 ± 271	608 ± 134
N4	15 ± 5	18 ± 14	271 ± 86	330 ± 105

5.4 Expression GFRα1, GFRα2, and Ret in human brain

Together, the data suggest NRTN variant N4 to be biologically active in vitro in the presence of either GFRα1/Ret or GRFα2/Ret complex, and to have therapeutic potential in vivo in an animal model of PD.

To find out whether these results can be translated to human trials, RT-PCR analysis was carried out to detect needed receptor components in the target tissue. Our analysis demonstrated GFRα1, GFRα2, and Ret to be present in human SNpc, globus pallidus, and putamen. This is in line with previously reported data, where GFRα1 and Ret have been reported to be detected in human putamen both in healthy elderly and in PD patients (Bäckman et al., 2006). Our findings further support further the translational value of the novel NRTN variants.

5.5 GDNF isoforms

GDNF is one of the most studied neurotrophic factors. Although the neuroprotective and neurorestorative properties of the α-isoform are well studied (reviewed in Björklund et al., 1997), only little is known about the biological activity of the shorter β-isoform. In the third study, the focus was on the effects of overexpressed β-GDNF in the non-lesioned striatum and in the partial 6-OHDA lesion model of PD.

5.5.1 scAAV1-mediated overexpression of GDNF isoforms in non-lesioned striatum

GDNF isoforms were overexpressed in the striatum with the self-complementary adeno associated virus, serotype 1 (scAAV1) vector. The viral vectors carrying either eGFP, α-GDNF or (β-GDNF) were injected into three striatal sites (coordinates in table 4). Striatal samples were collected three weeks later to determine the level of GDNF overexpression with enzyme-linked immunosorbent assay (ELISA) (figure 7C). The level of GDNF was significantly increased in α-GDNF and β-GDNF treated animals compared to GFP-treated animals (Kruskal-Wallis H(3)=15.457, p=0.001. Bonferroni post hoc test GFP vs α-GDNF p=0.005, GFP vs β-GDNF p=0.017, table 11). GDNF-immunoreactivity was spread widely around the injected hemisphere, whereas the GFP-staining pattern was more restricted. In either case, the immunoreactivity was observed only in the injected side, and the non-injected side was devoid of staining signal (study III, figure 2B). In addition, immunofluorescence staining from the SN of the GFP-treated animals showed the scAAV1 vector to transduce local medium spiny neurons projecting to SNpr (study III, figure 2C).

Table 11. Effects of GDNF isoforms in non-lesioned striatum.

Treatment	ELISA[a] [GDNF] pg/mg tissue	TH-ir % of the intact side	DAT-ir % of the intact side	D-amphetamine-induced Fos-ir % of the intact side
eGFP	15 ± 6	111 ± 4	104 ± 3	105 ± 4
α-GDNF	1906 ± 629**	124 ± 7	124 ± 4***	102 ± 4
β-GDNF	1115 ± 402*	115 ± 6	122 ± 3**	108 ± 3

[a]Intact side 40 ± 8 pg/mg tissue

*p<0.05 (vs GFP), **p<0.01 (vs GFP), ***p<0.001.

DAT-ir, dopamine transporter-immunoreactivity; ELISA, enzyme-linked immunosorbent assay; Fos-ir, Fos-immunoreactive cells; TH-ir, tyrosine hydroxylase-immunoreactivity.

Overexpression of GDNF did not affect the density of striatal TH-immunoreactive fibers (table 11). In contrast, both GDNF isoforms increased the optical density of DAT-immunoreactive fibers (one-way ANOVA $F_{2,24}$=11.336, p<0.001, Fisher's LSD post hoc test α-GDNF vs GFP p<0.001, β-GDNF vs GFP p=0.002, table 10). When the striatum was divided to rostral, central and caudal sections to have more detailed analysis, the GDNF isoforms effect was found to be consistent throughout the whole striatum (two-way ANOVA treatment effect $F_{2,72}$=23.285, p<0.0001; site effect $F_{2,72}$=0.490, p=0.615; treatment x site effect $F_{4,72}$=0.588, p=0.672. One-way ANOVA rostral $F_{2,24}$=5.315, p=0.012, Fisher's LSD post hoc analysis α-GDNF vs GFP p=0.005, β-GDNF vs GFP p=0.026; central $F_{2,24}$=11.339, p<0.0001, Fisher's LSD post hoc analysis α-GDNF vs GFP p<0.0001, β-GDNF vs GFP p=0.002; caudal:$F_{2,24}$=7.674, p=0.003 Fisher's LSD post hoc analysis α-GDNF vs GFP p=0.001, β-GDNF vs GFP p=0.006).

β-GDNF has been suggested to be secreted activity-dependently upon overexpression in vitro (Lonka-Nevalaita et al., 2010). We tested whether administration of amphetamine sulfate would also increase the secretion of β-GDNF in vivo. The possible increase in the secreted GDNF was measured indirectly using Fos as a marker for neuronal activity. The number of Fos-immunoreactive cells in the striatum was assessed two hours after amphetamine sulfate administration (2.5 mg/kg, s.c.). However, this hypothesized effect was not observed as the number of Fos-immunoreactive striatal cells was at the same level in all treatment groups (table 11). This might be due to low number of animals in the experiment (n=5) and short follow-up time after d-amphetamine administration.

5.5.2 Neuroprotective effects of GDNF isoforms in partial 6-OHDA rat model of PD

Neuroprotective effects of GDNF isoforms were assessed by inducing a partial lesion of the nigrostriatal tract three weeks after viral vector administration. The lesion was induced by injecting 3 x 2 µg of 6-OHDA to the striatum of the animals as designated in table 4. Effects were followed with drug-free cylinder test, amphetamine-induced rotations, and immunohistochemistry (figure 7D). Cylinder test was performed before lesioning on week three and four weeks after 6-OHDA injection. In the pre-lesion cylinder, GDNF-treated animals exhibited increased exploratory activity (one-way ANOVA $F_{2,43}$=3.871, p=0.028, Fisher's LSD post hoc test GFP vs α-GDNF p=0.019, GFP vs β-GDNF p=0.024, table 11). 6-OHDA-induced degeneration of the nigrostriatal tract attenuated this effect, and the activity of all treatment groups was at similar level four weeks later (two-way ANOVA treatment effect $F_{2,86}$=3.406, p=0.038; 6-OHDA effect $F_{1,86}$=29.071, p<0.0001; treatment x 6-OHDA interaction $F_{2,86}$=1.130, p=0.328, table 12). Although both GDNF isoforms induced the exploratory activity in pre-lesion cylinder test on week three, only α-GDNF increased the use of the contralateral (left) paw (one-way ANOVA $F_{2,43}$=4.492, p=0.017, Fisher's LSD post hoc test GFP vs α-GDNF p=0.005, table 11). However, this effect was abolished by 6-OHDA injection, as the use of contralateral paw was at a similar level in all treatment groups four weeks later (Two-way ANOVA treatment effect $F_{2,86}$=3.215, p=0.045; 6-OHDA effect $F_{1,86}$=41.803, p<0.0001; treatment x 6-OHDA interaction $F_{2,86}$=0.545, p=0.582, table 11). The results from the rotation assay are in line with the cylinder test results since no differences were observed between the treatment groups at either time point. However, β-GDNF treated animals did rotate slightly less than GFP- or α-GDNF treated animals on both time points, but the difference was not significant.

Table 12. Effects of GDNF isoforms on behavioral correlates.

Treatment	Cylinder Contralateral paw (%)		Cylinder activity (number of rearings)		Rotations (120min)	
	Pre-lesion Week 3	Post-lesion Week 7	Pre-lesion Week 3	Post-lesion Week 7	Post-lesion Week 5	Post-lesion Week 7
eGFP	48 ± 6	18 ± 6	36 ± 3	28 ± 3	466 ± 111	628 ± 168
α-GDNF	70 ± 5**	28 ± 9*	46 ± 4	30 ± 3	530 ± 635	635 ± 188
β-GDNF	56 ± 4	26 ± 8*	46 ± 2	32 ± 2	292 ± 82	427 ± 82

*p<0.05 (vs GFP) **p<0.01 (vs GFP)

Neuroprotective effects of GDNF isoforms were also evaluated with immunohistochemistry. Seven weeks after viral vector injection and four weeks after 6-OHDA administration the density of striatal TH-immunoreactive fibers was at a similar level in all treatment groups (table 13). Results from the DAT-immunoreactive fiber density were similar with TH, all treatment groups were at similar level. However, when DAT-immunoreactivity pattern was evaluated in more detail by dividing striatum to rostral, central, and caudal sections, a significant treatment effect was detected (treatment effect $F_{2,85}$=4.388, p=0.015; site effect $F_{2,85}$=0.272, p=0.762; treatment x site interaction $F_{4,85}$=0.130, p=0.971. One-way ANOVA rostral $F_{2,28}$=1.358, p=0.274; central: $F_{2,28}$=1.045, p=0.375; caudal: $F_{2,29}$=2.343, p=0.114).

Table 13. Immunohistochemical correlates of GDNF isoforms after 6-OHDA challenge.

Treatment	TH-ir str % of the intact side	DAT-ir str % of the intact side	TH-ir SNpc % of the intact side
eGFP	31 ± 7	26 ± 7	33 ± 7
α-GDNF	35 ± 6	44 ±11	56 ± 4***
β-GDNF	43 ± 7	49 ± 9	57 ± 4***

***p<0.001 (vs GFP).

DAT-ir, dopamine transporter-immunoreactivity; SNpc, substantia nigra pars compacta; str, striatum; TH-ir, tyrosine hydroxylase-immunoreactivity.

In SNpc, both isoforms increased the number of TH-immunoreactive cells after 6-OHDA-induced degeneration (One-way ANOVA $F_{2,42}$=8.828, p<0.001; Fisher's LSD *post hoc* test GFP vs α-GDNF p=0.001, GFP vs β-GDNF p<0.001, table 12). When the effect was evaluated in more detail, dividing SNpc to rostral, central, and caudal sections, the effect was found to be consistent throughout the whole SNpc (Two-way ANOVA treatment effect $F_{2,124}$=21.493, p<0.001; site effect $F_{2,124}$=0.388, p=0.679; treatment x site interaction $F_{4,124}$=0.352, p=0.842. One-way ANOVA rostral $F_{2,42}$=6.004, p=0.005, Fisher's LSD post hoc analysis α-GDNF vs GFP p=0.003, β-GDNF vs GFP p=0.008; central $F_{2,41}$=8.784, p=0.001, Fisher's LSD post hoc analysis α-GDNF vs GFP p=0.004, β-GDNF vs GFP p<0.0001; caudal $F_{2,41}$=7.214, p=0.002 Fisher's LSD post hoc analysis α-GDNF vs GFP p=0.001, β-GDNF vs GFP p=0.004).

Careful inspection of the rostral striatal TH- and DAT-stained sections revealed both GDNF isoforms to induce sprouting of TH- and DAT-immunoreactive fibers in globus pallidus seven weeks after scAAV1-vector injections. Globus pallidus of the GFP-treated animals was completely devoid of TH- or DAT-immunoreactivity. Sections from non-lesioned animals from an earlier time point, three weeks after viral vector administration, did not show TH- nor DAT-immunoreactive sprouting in globus pallidus.

6 DISCUSSION

This work was planned to explore the neuroprotective effects of novel neurotrophic factor variants in the rat partial 6-OHDA model of PD. Moreover, the new low-dose 6-OHDA models of PD were characterized together with a new method to evaluate the number of TH-positive cells in SN. Together the results show how low-doses of 6-OHDA can be used to induce a reliable animal model of PD and that the less known and less studied variants of neurotrophic factors have neuroprotective potential and are therefore new candidates for PD drug development.

6.1 Matlab-based cell counting

Stereology has traditionally been the method of choice for evaluating the number of TH-positive cells in SNpc (Sterio, 1984). Commonly the cell counting has been conducted using a three-dimensional probe called an optical dissector, which allows the cell number to be estimated regardless of their shape, size, and orientation, and the fractionator method, which is a systematic and uniform sampling pattern enabling every part of the sample to be count with an equal probability. The combination of these two principles in cell counting results in highly efficient and unbiased estimates of cell numbers (Sterio, 1984; West, 1999). The downside of using this well-established method is that it is very time-consuming. Therefore, we developed a new, algorithm-based method for estimating the cell numbers (study II). There are several critical differences between these two methods. The stereology-based method is used with a microscope, allowing to focus on different layers of the sample (which in this case is a 40 µm thick section), and the cells are counted three-dimensionally from the actual brain sections. With the algorithm-based method, the cells are counted two-dimensionally from the digitalized images taken from the sections. Thus, the quality of images influences the quality of the analysis and therefore we have used the automated slide scanner to obtain high-quality images. Moreover, in the algorithm-based method, the program recognizes the cells based on their signal intensity and size in pixels, unlike in the stereology-based method, where the user recognizes the cells. Yet another difference is how these two methods take into account the space between the sections. In this study, the 40 µm thick tissue sections were cut in series of six, thus the distance between two adjacent sections was 240 µm. StereoInvestigator, the computer program used to count cells with stereology, also estimated the number of cells between the sections, thus providing a cell number estimate for the whole SNpc. The Matlab algorithm did not perform this calculation and only the cell numbers per section were obtained. However, this might be correctable as the algorithm used for cell counting is freely available and easy to modify. Due to these differences a lot of information is lost while counting cells with our algorithm-based method, and consequently the obtained cell numbers are lower than the estimates obtained with stereology-based methods. Despite these differences between the methods, there is a good correlation between the results obtained with both of these methods (study II). It would be interesting to test this Matlab algorithm-based method in thin paraffin sections (~5 µm thick) and compare the results with stereology, as the thin sections contain only one cell layer. In this case, the cells would be counted two-dimensionally in the section with both methods and less information would be lost with Matlab. Hence, one could expect to have an even higher correlation between these two methods.

6.2 6-OHDA model of Parkinson's disease

Since 6-OHDA does not cross the blood-brain barrier, it has to be administered to the brain parenchyma, either to SNpc, where the dopaminergic cell bodies are located, MFB, through which the nigrostriatal tract ascends, or striatum, where the nerve terminals are located (Duty and Jenner, 2011). Whereas injection of 6-OHDA to MFB or SN results in an almost complete (80-90%) loss of nigral TH-positive cells within one week (Ungerstedt, 1968; Jeon et al., 1995; Zuch et al., 2000), injection of 6-

OHDA to the striatum produces a more slowly progressive partial loss of nigral cells (Sauer and Oertel, 1994; Lee et al., 1996; Kirik et al., 1998). Nerve terminals start to degenerate during the first 24 hours after the striatal administration of 6-OHDA, and the lesion is fully developed within the next four weeks (Sauer and Oertel, 1994; Lee et al., 1996; Blandini et al., 2007). Thus, the striatal 6-OHDA injection model is in line with the current "dying back" theory of PD (Burke and O'Malley, 2013), where the loss of striatal dopaminergic nerve terminals outweighs the loss of nigral cell bodies. It has been estimated that there is about 30% (adjusted for age) loss of dopaminergic neurons in the SNpc at the time of symptom onset in PD patients (Fearnley and Lees, 1991; Cheng et al., 2010). In the striatum, there would be 50-70% loss of dopaminergic nerve terminals and 70-80% decrease of dopamine (Cheng et al., 2010; de la Fuente-Fernandez, 2013). This further supports the use of the striatal model in testing new therapeutic agents for PD, since it more closely resembles the early stage of PD, unlike MFB or SN lesion models, which mimic later stages of PD. On the other hand, MFB and SN models might be more suitable to study the mechanisms of cellular degeneration. Moreover, dorsal striatal targeting of 6-OHDA might provide the most appropriate model of PD in rodents. The dorsal part of the striatum might be the equivalent of the putamen in human and primate brain, as it receives input from motor and sensory neocortical areas in addition to dopaminergic innervation from SN (Björklund et al., 1997; Kirik et al., 1998; Deumens et al., 2002), and the dopamine depletion is most pronounced in the putamen in PD patients (Kish et al., 1988).

In study I we used 1 x 28 µg 6-OHDA dose. In the experiment only 54% of the animals had developed a lesion in two weeks (table 7). Thus, a large number of animals was needed to inject with 6-OHDA to have sufficient number of animals in each treatment groups for study I. The reason for the low success rate might be the injection paradigm itself. 1 x 28 µg lesions were done as four depots to same injection tract (table 4), where the microinjection needle was lifted between each depot and this might have caused the 6-OHDA to flow back up the needle tract (Butcher, 1975). When compared with other used 6-OHDA administration paradigms (listed in table 4), the success rate was higher when the 6-OHDA injections were done as single depot per injection tract (table 7). This is also more common way of injecting 6-OHDA (e.g. Lee et al., 1996; Kirik et al., 1998; Bäck et al., 2013b). The advantage of high success rate is that less animals is needed to produce reliable data. Interestingly, in the first measured time point the highest success rates were in lesions classified as regressive (2 x 10 μg^{reg} and 1 x 20 µg), in both groups all animals rotated over 100 turns. However, in the last time point, only 43% of the animals in 2 x 10 μg^{reg} group rotated over the 100 turn limit. In contrast in progressive lesion groups (3 x 3 µg, 2 x 10 μg^{prog}, and 3 x 7 µg) the success rates were high in both time points. This comparison supports the use of multiple injection sites for creating a 6-OHDA lesion model of PD with success rate.

Stability of the animal model is crucial as it enables longer experiments and sufficient follow-up periods for neurorestoration and neuroprotection experiments, reduces the number of animals needed for the experiments and more importantly, increases the reliability and replicability of the results. The 6-OHDA lesion groups were categorized as progressive, stable or regressive based on their rotational behavior. In progressive lesion groups the rotational behavior of the animals increased in time (3 x 3 µg, 2 x 10 μg^{prog}, and 3 x 7 µg), in stable groups the rotational behavior was at similar level during the whole experiment (3 x 1 µg, 3 x 2 µg, and 1 x 28 μg^{2week}, and 1 x 28 μg^{5week}), and in regressive groups (2 x 10 μg^{reg} and 1 x 20 µg) the rotational behavior decreased in time (figure 8). Several studies have reported spontaneous recovery from the 6-OHDA lesion (Yang et al., 2009; Bäck et al., 2013a; Bäck et al., 2013b). Spontaneous recovery has been thought to occur by several mechanisms. In the lesioned striatum dopamine is diffused more widely (Robinson et al., 1994) and the amount of dopamine released from the remaining nerve terminals is increased (Doucet et al., 1986). These presynaptic compensatory mechanisms take place almost immediately after the 6-OHDA injection, and together with axonal regrowth can provide long-lasting effects (Robinson et al., 1994; Blanchard et al., 1996). In our hands, injection of low doses of 6-OHDA to multiple sites seemed to improve the stability of the lesion (figure 8, table 7).

One possible explanation for the high rotation rates at the last time point of the progressive lesion groups might be sensitization to amphetamine. Considering that the experiments are often long, and the rotation assay is repeated several times, the animals are repeatedly administered amphetamine in a drug-paired environment. This can lead to increased locomotor activity, i.e. the animals rotate without amphetamine (Robinson and Becker, 1986; Kalivas and Stewart, 1991). This conditioned response has also been detected in MFB-lesioned rats with apomorphine (Hudson et al., 1994). To discriminate the conditioned response from the actual drug-induced rotations, we have implemented a 30 minute habituation period before administration of amphetamine. We also decreased the dose of amphetamine from previously used 2.5 mg/kg (Voutilainen et al., 2009; Bäck et al., 2013a; Bäck et al., 2013b) to 2.5mg/kg of amphetamine sulfate, which equals to 1.84 mg/kg of d-amphetamine, and changed administration from intraperitoneal to subcutaneous. Due to these changes, only one rotation peak was observed in the assay, despite the time point. However, in our experiments the progressive lesion groups were assessed approximately as many times as stable or regressive groups, and therefore the sensitization to amphetamine is less likely to explain the differences in rotational behavior.

As discussed above an in section 2.2.1, amphetamine-induced rotations might not be the optimal method to assess the lesioned animals. In studies I and III we used cylinder test together with the rotational assay to determine the therapeutic effects of neurotrophic factors In our hands, the problem with cylinder test is the habituation. As the test is based on the exploratory activity, the rats habituate to the environment when the test is repeated and this is detected as a reduction in the vertical activity. To avoid this problem, in study III the cylinder test was conducted under red light. This seemed to increase the activity of the animals, and a reasonable number of rearings were measured during five minutes of testing (GFP-treated animals week 3 36 ±3 rearings and week 7 28 ± 3 rearings).

The classical method for determining the magnitude of the lesion has been counting of nigral TH-immunoreactive cells and striatal density of TH-immunoreactive fibers (Sauer and Oertel, 1994; Bowenkamp et al., 1996; Björklund et al., 1997; Cohen et al., 2011). Third alternative to use as a marker for dopaminergic neurons is VMAT2 (Decressac et al., 2011; Kumar et al., 2015). Immunohistochemistry is a not fully problem-free approach because the cells first become atrophic and lose their dopaminergic phenotype before degenerating (Sauer and Oertel, 1994; Bowenkamp et al., 1996; Björklund et al., 1997; Cohen et al., 2011). One way of distinguishing these two events, actual cell degeneration and loss of TH-phenotype in nigral cell bodies, is to label dopaminergic cells with fluorescent retrograde axonal tracer FluoroGold (Sauer and Oertel, 1994; Bowenkamp et al., 1996; Cohen et al., 2011). The loss of TH-immunoreactivity has been shown to be maximal about two weeks after 6-OHDA administration, whereas FluoroGold-signal decreases more slowly, until week four. The discrepancy between the number of TH-immunoreactive cells and FluoroGold-positive cells is reduced in time when the lesion has developed properly (Sauer and Oertel, 1994; Cohen et al., 2011). Although in our experiments (studies II and II) the immunohistochemical analysis was conducted several (8-16) weeks after 6-OHDA administration, and thus the lesion was fully developed and TH-staining could be considered as a reliable marker for the dopaminergic cells (Sauer and Oertel, 1994; Lee et al., 1996; Cohen et al., 2011), another marker, DAT, was also applied to determine the lesion size. In study III this was a crucial step since long-term overexpression of GDNF is known to downregulate TH expression (Georgievska et al., 2002a; Georgievska et al., 2004a; Georgievska et al., 2004b). In all our stable lesion models tested (3 x 1 µg, 3 x 2 µg, and 1 x 28 μg^{2week}), the loss of TH-immunoreactivity was more pronounced in the striatum compared to SN (study I, II, table 7), suggesting these models to be more appropriate in modelling the earlier stage of PD and testing new neuroprotective agents.

The effect of 6-OHDA dose on the lesion specificity has been well documented, and high doses of 6-OHDA have been shown to induce gliosis and unspecific loss of neurons around the injection tract (Przedborski et al., 1995; Lee et al., 1996; Kirik et al., 1998), where the local 6-OHDA concentration is highest (Agid et al., 1973; Butcher, 1975; Javoy et al., 1976) and enlargement of the ipsilateral ventricle (Przedborski et al., 1995). Therefore the use of low doses in inducing the lesion is highly recommended

(Kirik et al., 1998). Another thing to consider to minimize the unspecific damage due to injection is the size of microinjection needle used to infuse 6-OHDA to brain parenchyma. In study II we calculated that one injection with 26 G needle (outer diameter 0.47 mm) caused an approximately 2.6% mechanical lesion in the striatum. In contrast, one injection with a smaller 33G microinjection needle (outer diameter 0.21 mm) caused 0.5% mechanical lesion. The choice of the needle size becomes even more crucial when the injections are done into multiple sites in brain or to a smaller target (i.e. MFB or SN) or to a smaller animal (i.e. mouse). On the other hand, smaller needle might clog more easily. The third thing to consider for minimizing the mechanical damage is the infusion rate of the toxin. Fulceri and coworkers (Fulceri et al., 2006) have shown how slower 6-OHDA infusion speed spared the noradrenergic nerve terminals around the needle tract from degeneration. Although 6-OHDA is taken up to the cells via DAT, it has a high affinity for the noradrenalinetransporter (Luthman et al., 1989). Therefore, noradrenaline transporter blockers, such as desipramine (desmethylimipramine), can be administered prior to 6-OHDA injection to reduce the unspecific damage (Fulceri et al., 2006; Bäck et al., 2013a). Moreover, if the experimenter desires that the SNpc stays intact and devoid of mechanical damage, the 6-OHDA should be injected to either MFB or striatum.

To summarize, we have tested several different injection paradigms to find the optimal settings to induce reliable partial 6-OHDA lesion model of PD with high success rate. Stability and high success rate of the model would reduce the number of animals needed in the experiments to produce reliable data. After the comparison and for the aforementioned reasons we chose the low 3 x 2 µg dose of 6-OHDA for the study III. This lesion paradigm produced stable rotational behavior for twelve weeks with good success rate, and showed more pronounced loss of TH- or DAT-immunoreactivity in the striatum compared to SNpc. However, similar doses as were used in our comparison have been used in successfully in experiments elsewhere (Przedborski et al., 1995; Kirik et al., 1998). Thus, there are several technical details which can affect the reliability of 6-OHDA model of PD.

6.3 NRTN variants

NRTN and its homolog GDNF both bind to heparan sulfated proteoglycans of the cell surface and extracellular matrix, hampering protein diffusion in the brain tissue (Hamilton et al., 2001; Bespalov et al., 2011). Since neurotrophic factors are secreted in small quantities, binding to cell surface might be used to concentrate them near their receptors (reviewed in Sariola and Saarma, 2003). However, this low diffusion has been suggested to be one of the reasons for the mixed results in clinical trials (Salvatore et al., 2006; Bartus et al., 2015). Moreover, some animal studies have suggested infused NRTN to precipitate in tissue resulting in high focal concentration around the infusion site (Hadaczek et al., 2010). To overcome these problems and to improve the diffusion capacity in brain parenchyma, novel NRTN variants were designed.

The structure of NRTN was modeled based on the known structure of GDNF (Eigenbrot and Gerber, 1997; Parkash et al., 2008), and mutations were introduced to the heel region, where the putative heparin-binding site (Cardin and Weintraub, 1989) was located. In the N4 variant, the heel region sequence was replaced with the heel sequence from persephin, which does not bind heparin (Bespalov et al., 2011). However, the diffusion properties of persephin in the brain tissue have not been studied in detail. In GDNF the finger regions are more important for the interaction between the neurotrophic factor and the GFRα receptor than the heel region (Eketjäll et al., 1999; Parkash et al., 2008), thus supporting the choice of mutation site. Mutation in the heel region probably did have a small effect on the orientation of the finger structures which are interacting with GFRα receptors, but it did not affect the biological activity of the variant proteins. However, all NRTN variants have stronger affinity for GFRα2 than for GFRα1, which has been shown previously for WT NRTN (Cik et al., 2000).

Based on the observation of Hoane and colleagues (Hoane et al., 2000) that WT NRTN produced in mammalian cells is more biologically active in vitro than WT NRTN produced in E. coli, NRTN variants

were decided to produce in mammalian chinese hamster ovary cells. The difference in biological activity could be explained by post-translational modifications and better quality control of the secreted proteins. NRTN variants demonstrated biological activity in several in vitro assays. Interestingly, although WT NRTN is not able to induce extraureteric budding in a kidney ex vivo organogenesis assay, NRTN variants N2 and N4 both induced ectopic ureteric budding and swelling of the buds. Higher biological activity in the assays correlates with higher stability of N2 and N4 in the organogenesis and cell culture media.

Since the composition of heparan sulfate proteoglycans of the extracellular matrix and cell surface might vary between cell types, animal species and their developmental stage (reviewed Sarrazin et al., 2011), the diffusion properties of NRTN variants were tested in three different species: rat, cynomolgus, and marmoset monkey. The affinity of N2 and N4 to heparin was at similar level in vitro, but in brain parenchyma N4 diffused better. One possible explanation for this discrepancy might be the difference in the structure of the variants. In N2 three point mutations were introduced to the heparin binding sequence, whereas in N4 the whole sequence was replaced. Furthermore, the environment of the in vivo assay is more complex than in vitro. Thus, the in vivo diffusion capacity reflected the neuroprotective effects of N4. The increased stability and diffusion capacity of NRTN variants could mean that these proteins reach a higher number of neurons and receptors, inducing the survival of axons and not only the somata, and this would be reflected by the ability of N4 to rescue striatal TH-immunoreactive nerve fibers. On the other hand, the accumulation of NRTN to the near vicinity of GFRα-receptors has been thought to improve signaling (reviewed in Sariola and Saarma, 2003). Maybe this is the case with GDNF, as elimination of heparin-binding sequence from the protein (Δ38N-GDNF) reduced the neuroprotective effects in the 6-OHDA model of PD (Piltonen et al., 2009). This is in contrast to NRTN variants, which showed better neuroprotective effects in the 6-OHDA model of PD than WT GDNF.

Although wide diffusion around the infusion site is desired to enhance the therapeutic effects, diffusion outside the target area (putamen / SN in PD) might cause side effects. As discussed above, the higher diffusion increases the number of reached target receptors, but simultaneously reduces the concentration of the therapeutic agent around the needle tract. Which one of the possibilities is more beneficial depends on the location of the target receptors. Besides diffusion properties and a number of infusion sites, the vasculature of the brain also affects the spreading of the protein in the brain. Hadeczek and coworkers (Hadaczek et al., 2006) have shown how arterial pulsation generated by cardiac contractions can affect the diffusion of intraparenchymally infused molecules in perivascular spaces around arteries and arterioles. Thus, understanding of the organization of the brain vasculature and perivascular fluid dynamics is of importance while optimizing the efficiency of therapeutic molecule transport and diffusion.

In a neuroprotection assay, where low doses of proteins (5 µg) were infused two weeks after 6-OHDA injections before the lesion had fully developed (Sauer and Oertel, 1994; Lee et al., 1996; Blandini et al., 2007), N4 was able to improve the motor performance both in cylinder test and in amphetamine-induced rotations. The results from both behavioral assays are in line, and remarkably, there is a clear result in cylinder test, which is a more robust test than amphetamine-induced rotations (Hudson et al., 1993; Kirik et al., 1998; Schallert et al., 2000). The behavioral results from the neurorestoration assay are less clear. This can be due to the low number of animals (n=6-7/group) but also due to the robust lesion used in the study. The neurotrophic factors were administered five weeks after 6-OHDA injection when the lesion is fully developed, and the high dose of 6-OHDA induced a severe lesion. However, together these results strongly suggest the improvement in the diffusion capacity to improve the neuroprotective effects of NRTN variants.

6.4 GDNF isoforms

Consistent with previous studies showing exogenous GDNF to increase the locomotor activity of the animals (Hudson et al., 1995; Hebert and Gerhardt, 1997; Kirik et al., 2000a), overexpression of GDNF isoforms in non-lesioned striatum induced the exploratory activity of the animals in the drug-free cylinder test. Interestingly, only α-GDNF increased the use of the contralateral paw. This finding is in accordance with the in vitro observation of different secretory patterns of GDNF isoforms (Lonka-Nevalaita et al., 2010). In this case, the constitutive secretion of α-GDNF and corresponding mature GDNF would have an effect on the behavior, whereas the β-GDNF and corresponding mature GDNF would be retained inside the cells and therefore no effect on the use of paws was observed. However, this would also suggest the increased activity of the animals and the use of paws to have different mechanisms.

Effects of GDNF on TH have been well-documented. A single bolus of GDNF increases TH expression (Hudson et al., 1995) and activity (Lapchak et al., 1997), whereas continuous overexpression of GDNF decreases striatal TH levels (Georgievska et al., 2002a; Georgievska et al., 2004b; Sajadi et al., 2005) as a response to long-lasting dopamine neuron activity (Iwata et al., 2000; Sajadi et al., 2005). In contrast, in non-human primates long-term overexpression of GDNF increases TH-immunoreactivity in the putamen (Kordower et al., 2000; Palfi et al., 2002; Johnston et al., 2009), suggesting the downregulation of TH to be a species-specific phenomenon. Nevertheless, the effects of GDNF on another important enzyme regulating the dopamine transmission, DAT, are still largely unknown. Data from Salvatore and colleagues (Salvatore et al., 2009) suggests dose-dependence, where low doses of GDNF protein would not have an effect on striatal DAT expression, but in higher doses GDNF downregulates DAT. GDNF might regulate the DAT activity by increasing dimerization and protein-protein interactions (Barroso-Chinea et al., 2016). Our results show overexpression of GDNF to have different effects on TH and DAT. In non-lesioned striatum, both isoforms increased the density of DAT-immunoreactive fibers but did not have an effect on TH-immunoreactive fibers (table 11). The increase of DAT could suggest GDNF-treated animals to be more vulnerable for 6-OHDA-induced cytotoxicity since 6-OHDA is taken up by DAT. This was observed with mice overexpressing GDNF from the native locus (Kumar et al., 2015). The mice have increased activity of DAT, but no increase in the DAT protein level, and increased number of striatal dopaminergic nerve terminals, and thus they are more susceptible to 6-OHDA cytotoxicity (Kumar et al., 2015). However, our data do not support this, the number of TH-immunoreactive cells in SNpc was significantly higher in α-GDNF- and β-GDNF-treated animals than in GFP-treated animals after 6-OHDA administration (table 13).

Several studies where the long-term expression of GDNF has been implemented with the help of viral vectors have been conducted, but the comparison and translation of the obtained results is difficult, since the results vary according to the used dose, administration sites, and used time points (reviewed in Björklund et al., 2000; Kirik et al., 2017). In striatum, GDNF functions as a chemoattractant, inducing axonal sprouting and reinnervation after a cytotoxic insult, and that might explain why striatal delivery of GDNF gene protects the nigrostriatal tract and provides functional recovery in the animal models of PD (Kirik et al., 2000b; Kells et al., 2010). However, neuroprotective effects of striatal GDNF delivery without behavioral correlates have also been reported (Georgievska et al., 2002b). This is in line with our results, where both GDNF isoforms protected TH-immunoreactive cell bodies from 6-OHDA-induced degeneration without functional recovery. Nonetheless, using a similar experimental setup (striatal administration of AAV-GDNF four weeks before striatal infusion of 6-OHDA) but with a longer follow-up time, Kirik and coworkers (Kirik et al., 2000b) showed that the GDNF-treated animals rotated less with amphetamine four weeks after 6-OHDA lesion, but the difference was not significant before week seven. Therefore the short four-week follow-up time used in our study might explain the lack of functional effects.

In the experiment the transgene was expressed under cytomegalovirus (CMV) promoter, which has been shown to drive the transgene expression both in neurons and astrocytes (Bockstael et al., 2008).

Although the titers of the used viral vectors were similar (scAAV1-CMV-pre-α-pro-GDNF 2.14 x 10^{12} vg/ml and scAAV1-CMV-pre-β-pro-GDNF 1.73 x 10^{12} vg/ml), the GDNF level was much higher in α-GDNF overexpressing animals than in animals overexpressing β-GDNF. A similar phenomenon has been observed when Fletcher and coworkers used DNA nanoparticles to overexpress GDNF isoforms in the rat striatum (Fletcher et al., 2011). Furthermore, the expression of endogenous GDNF and exogenous GDNF delivered with DNA nanoparticles is enhanced by 6-OHDA injection (Yurek and Fletcher-Turner, 2001; Fletcher et al., 2011). Despite the difference in the protein levels, immunohistochemistry showed a widely spread GDNF-immunoreactivity pattern in both α-GDNF- and β-GDNF-treated animals. In comparison, GFP-immunoreactivity was detected in a more restricted area as it is retained inside the cells, unlike GDNF. Fluorescence staining with TH antibody revealed GFP to be expressed in SNpr and not in SNpc, suggesting scAAV1 to transduce GABAergic medium spiny neurons rather than dopaminergic neurons. However, this was not confirmed with GABAergic markers. This GFP-signal pattern is in line with previously published data with CMV promoter (Bockstael et al., 2008). The transport of transgene product to SNpr and the observed increase in the number of nigral TH-immunoreactive cells in GDNF-treated animals could imply to the paracrine function of GDNF. This might be a better alternative than nigral delivery of GDNF, which has been shown to induce aberrant sprouting (Kirik et al., 2000b; Georgievska et al., 2002a).

Recently, cytotoxic effects of high titers of AAV5-GFP was reported (Xilouri et al., 2013; Landeck et al., 2017). In their study Landeck and colleagues showed that nigral administration of high titer (3.3 x 10^{14} gc/ml) AAV5-GFP induced loss of TH-immunoreactive cells in SNpc. In our case, the used titers were lower (7.4 x 10^{13} vg/ml) and no cytotoxic effects were detected after intrastriatal delivery since the loss of TH-immunoreactive cells was about 67% in AAV-GFP-treated animals (study III) and 64% in animals receiving only 3 x 2 µg dose of 6-OHDA (study II). Cytotoxicity was not observed in striatum either since the loss of TH-immunoreactivity was 72% after 6-OHDA injection in study II (table 7), and 69% after the combination of AAV-GFP and 6-OHDA in study III (table 13).

6.5 Protein infusion vs gene therapy

As mentioned earlier, the focus in neurotrophic factor clinical trials is currently on increasing the target coverage of the therapeutic protein. This can be achieved by improving the bioavailability of neurotrophic factors in the target area by increasing the infusion volume and/or dose, modifying the vector, or by modifying the diffusion properties of the therapeutic agents. Comparison of the obtained data is difficult since the results depend on the used model (ex. whether 6-OHDA was injected to MFB, SN, or striatum), time frame, when was the neurotrophic factor infused (e.g. before or after 6-OHDA) and how, as protein infusion or with viral vectors. This variability makes it more difficult to translate the results. In this thesis, the bioavailability of NRTN in the target tissue was increased by modifying the protein sequence (study I) and the bioavailability of GDNF isoforms by viral vector delivery (study III).

Gene therapy provides long-lasting expression of transgene in the target tissue. However, with viral vectors the dosing is more difficult because the amount of therapeutic protein produced depends on the virus vector properties and used promoter (reviewed in Domanskyi et al., 2015). In addition, once the vector has been infused to parenchyma, it cannot be removed. Regulated viral vectors, such as the ones using TetON/OFF promoters, destabilizing domains, or GeneSwitch, where the expression of the transgene can be turned on or off with antibiotics or small molecules such as doxycycline or trimethoprim, might solve this problem (Maddalena et al., 2013; Quintino et al., 2013; Chtarto et al., 2016). High neurotrophic factor doses might not be desirable though, not only due to risk of side effects but also because neurotrophic factors are known to be effective in low concentrations (Sariola and Saarma, 2003).

With protein infusion, the dosing may be easier to control than with viral vectors, and in case of side effects the administration can be stopped. However, the question arises whether the infusion should be continuous or repeated. As discussed above in section 6.3, in study I we used proteins produced in mammalian cells, as WT NRTN produced in mammalian cells was previously shown to be more active in vitro than the E. coli produced protein, probably due to post-translational modifications (Hoane et al., 2000). Thus, the manufacturing process of the proteins used in the protein infusion studies needs to be taken into account when comparing the obtained results. In study I we showed that by improving the diffusion capacity of NRTN we were able to enhance the therapeutic effects. Although the improved diffusion is a desired property, too diffuse protein might spread outside the target area and induce unwanted side effects.

Another important consideration is the targeting. Although nigral delivery of neurotrophic factors has been shown to be neuroprotective (Kirik et al., 2000a; Kirik et al., 2000b), overloading the stressed cells with proteins might accelerate the degeneration process instead of delaying it (Hoffer and Harvey, 2011). Moreover, nigral overexpression of GDNF can induce aberrant sprouting (Kirik et al., 2000b; Georgievska et al., 2002a). On the other hand, target-derived expression of neurotrophic factors is an attractive approach, as retrograde transport of GDNF has been reported (Tomac et al., 1995b; Ai et al., 2003). However, in the light of current evidence the degeneration process starts from the nerve terminals compromising the axonal transport (Kordower et al., 2013; Tagliaferro and Burke, 2016). Interestingly, as shown in study III, proteins expressed in striatal medium spiny neurons were transported anterogradely to SNpr, and hence striatally delivered neurotrophic factors may execute their functions on dopaminergic neurons in SNpc in a paracrine manner.

Ergo, there are numerous factors to consider in the development of efficient neurotrophic factor-based therapy. Besides focusing on developing an efficient and safe therapy, attention should be paid to find new diagnostic tools to identify PD earlier. Detection of early stage PD patients would allow earlier initiation of the treatment before most of the striatal nerve terminals and nigral cell bodies have degenerated and there are still viable cells left to respond to the therapy.

7 CONCLUSIONS

The aim of the thesis was to study the neuroprotective effects of novel and less studied variants of neurotrophic factors GDNF and neurturin. The main conclusions from the studies are the following:

- Reduced affinity to heparin in vitro results in improved diffusion capacity and enhanced therapeutic efficiency of neurturin variants in vivo to protect nigrostriatal dopaminergic circuitry.

- The stability of the classical striatal 6-OHDA model of PD can be improved by distributing 6-OHDA to multiple injection sites. In this way, even small doses (6-9µg) can produce a stable animal model with high success rate thus reducing the number of experimental animals.

- Both conserved GDNF isoforms increased the density of DAT-immunoreactive fibers in non-lesioned striatum and increased vertical activity of the animals, but only α-GDNF induced behavioral asymmetry. Both isoforms protected nigral dopaminergic neurons form 6-OHDA-induced cytotoxicity without behavioral improvements.

Together, the results point that these new isoforms of well-studied neurotrophic factors have therapeutic potential in PD. Improved diffusion properties of NRTN variants and regulated secretion of β-GDNF make them interesting new agents in the search for safe and efficient neurotrophic factor-based therapy.

8 ACKNOWLEDGEMENTS

This work was conducted at the Insitute of Biotechnology, University of Helsinki in collaboration with Division of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, University of Helsinki during the years 2012 to 2017.

Finnish Parkinson Foundation, Finnish Culture Foundation, Instrumentarium Science Foundation, Oskar Öflund Foundation, Finnish Pharmacological Society, Drug Research Foundation, and the Doctoral School of Health together with its Doctoral Programme in Drug Research are gratefully acknowledged for the personal financial support.

I want to thank my supervisor, docent Mikko Airavaara for the courage to hire a PhD student without any background in neuroscience. It's been quite a journey and I sincerely thank you for giving me this opportunity.

Professor Raimo Tuominen, Head of the Division of Pharmacology and Pharmacotherapy, is acknowledged for the support, optimism, and all the signatures during these years.

The reviewers of my thesis, docent Sanna Janhunen and docent Teemu Aitta-aho are thanked for the valuable comments and suggestions regarding my thesis.

Privat docent Liliane Tenenbaum is acknowledged for agreeing to be my opponent in the public defence of this thesis.

Professor Mart Saarma is thanked for the support and providing the possibility to work in his laboratory.

I want to express my gratitude to all my co-authors, your (major) efforts are highly appreciated. Without you I wouldn't have any papers. Especially Pia, it's been a pleasure to work with you. The NRTN paper taught me what it means that a paper should tell a story.

All the past and present members of Team Airavaara and Saarma lab are acknowledged for creating a friendly work environment. Dear mutants and wildtypes, Emmi, my songwriting partner FroBro/Franci/FNC, Jenni, Kat, Maryna, Polina, and Sara, I've enjoyed our adventures in and outside the lab enormously. Your support and friendhip means a lot to me.

All the students whom I have supervised and who have helped me in the lab: Antti Huovinen, Ulrika Julku, Jaan Korpikoski, Ilmari Parkkinen, and Aneta Petrušková, your help with stainings, cylinder videos and everything else is gratefully acknowledged.

Susanne Bäck and Kati Rautio at the Division of Pharmacology and Pharmacotherapy are acknowledged for all their help and support.

I want to thank my friends for the support and all the good times we've had together. I suck at staying in contact, but it doesn't mean that I have forgotten about you. Especially Laura, our "culture events" and evening teas have been great escapes from lab and other matters.

Anu & Aki, Aki, Laura, Selja & Siiri, Anne, Juha & Venla, I love the twisted humor we have in our family. Things are never as bad as they seem and they could always be a lot worse. Thank you for counterbalancing my "science life". Niin ja oot äiti sinäki semmone kapine.

Mission accomplished.

Helsinki, August 2017

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